

JLCI/SAS:amc 10/30/08

Declaration_Final (67021-06) E-184-2002/0-US-03

Attorney Reference Number 4239-67021-06

Application Number 10/533,634

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Ivins *et al.*

Application No. 10/533,634

Filed: April 29, 2005

Confirmation No. 5041

For: METHOD OF PREVENTING
INFECTIONS FROM BIOTERRORISM
AGENTS WITH
IMMUNOSTIMULATORY CPG
OLIGONUCLEOTIDES

FILED VIA EFS

Examiner: Emily M. Le

Art Unit: 1648

Attorney Reference No. 4239-67021-06

FILED VIA THE ELECTRONIC FILING SYSTEM
COMMISSIONER FOR PATENTS

DECLARATION UNDER 37 C.F.R. § 1.132

1. I, Dennis M. Klinman, M.D., Ph.D., am an inventor named in the above-referenced patent application. I am currently Head of the Immune Modulation Group at the National Cancer Institute of the National Institutes of Health (NIH). I have been employed by the Food and Drug Administration or the NIH continuously since 1983 and have conducted research in the fields of virology, immunology and vaccine development over that 25 year period. I have published more than 250 scientific articles in the fields of virology, immunology and vaccine development. A copy of my current curriculum vitae is submitted herewith.

2. I have read and understood the above-referenced patent application, including the pending claims, and the Office action dated July 31, 2008.

3. It is my understanding that in the Office action dated July 31, 2008, claims 37, 38, 40, 41, 50, 52-57 and 61-64 were rejected as allegedly obvious over Ivins *et al.* (*Eur. J. Epidemiol.* 4(1):12-19, 1988), in view of Verthelyi *et al.* (*J. Immunol.* 168:1659-1663, 2002) and Jones *et al.*

(*Vaccine* 17:3065-3071, 1999). It is also my understanding that it is alleged that it would have been obvious to use the CpG oligonucleotide adjuvant of Jones *et al.* with the anthrax vaccine of Ivins *et al.* in view of the teaching of Verthelyi *et al.* that CpG oligonucleotides can be used as adjuvants in primates.

4. Based on my experience in the field of vaccine development, it is not possible to predict whether CpG oligonucleotides of the K-type or D-type class will enhance the immunogenicity of a specific vaccine without empirical data. This is evidenced by Verthelyi *et al.* 2002, cited by the Office, which teaches that D-type oligonucleotides, but not K-type oligonucleotides, are effective when used with a heat killed *Leishmania* vaccine (HKL V). Verthelyi *et al.* describe work performed in my laboratory in which K-type and D-type CpG oligonucleotides were tested as adjuvants for HKLV. As shown in Figure 5 of Verthelyi *et al.*, D-type CpG oligonucleotide effectively enhanced the immune response elicited by HKLV, as evidenced by a decrease in lesion size following challenge with metacyclic promastigotes. In contrast, K-type CpG oligonucleotide did not significantly alter lesion size relative to vaccine alone. Of note, our empirical studies showed that K-type oligonucleotides but not D-type oligonucleotides were highly effective adjuvants when used in combination with AVA (the licensed anthrax vaccine), a result that directly contrasts with their relative activity when combined with HKLV.

5. In addition, my laboratory conducted studies in which a K-type CpG oligonucleotide was tested as an adjuvant for whole, inactivated influenza virus (heat killed PR8 influenza virus HKF). In these studies, we compared the magnitude of the IgG anti-influenza antibody response and protection conferred to mice treated with HKF alone or in combination with 50 µg/ml K-type CpG oligonucleotide or 50 µg of the lipid adjuvant monophosphoryl lipid A (MPL). The results showed that adding MPL significantly improved (more than doubled) the serum anti-influenza antibody response ($p < 0.02$) and improved survival (100% survival, $p < 0.05$). In contrast, the K-type CpG oligonucleotide did not significantly increase anti-influenza antibody levels or survival ($p > 0.35$ for both parameters). This result is in stark contrast to the results obtained using a K-type CpG oligonucleotide (CpG 7909; SEQ ID NO: 200) in combination with the anthrax vaccine AVA in


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macaques. As described in the specification, administration of AVA + CpG 7909 resulted in a 17-fold increase in neutralizing antibody titer relative to administration of AVA alone. Furthermore, serum from macaques vaccinated with AVA + CpG 7909 significantly increased passive protection to mice challenged with *Bacillus anthracis*, relative to serum obtained from macaques vaccinated with AVA alone.

6. These studies demonstrate that it is not possible to predict if a particular class of CpG oligonucleotide will function as an effective adjuvant for a vaccine without experimental testing. Therefore, one of skill in the art would not have been able to predict that a CpG oligonucleotide having the nucleotide sequence of SEQ ID NO: 200 would enhance the immunogenicity of an anthrax vaccine. Furthermore, given the findings described in Verthelyi *et al.* and other studies performed in my laboratory on influenza vaccines, it would not have been expected that a K-type oligonucleotide, such as an oligonucleotide having the nucleotide sequence of SEQ ID NO: 200, would enhance the immunogenicity of an anthrax vaccine, such as AVA.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: OCT 30, 2008


Dennis M. Klinthan, M.D., Ph.D.

CURRICULUM VITAE
Dennis M. Klinman, MD/PhD

Home Address:

2 Candlelight Court, Potomac, MD. 20854

Work Address:

Bldg 567 Rm 205
Center for Cancer Research, National Cancer Institute, Frederick, MD
21702
(301) 228-4265 (Work)
(301) 846-6641 (FAX)
E mail: klinmand@mail.nih.gov

Date/Place of Birth:

October 9, 1954; Philadelphia, PA

Marital Status:

Married to Dr. Susan Leitman-Klinman
Three daughters

Education:

1976	BS, Saint Joseph's University
1978	Graduate Member, Cambridge University
1982	MD/PhD, University of Pennsylvania
1987	Rheumatology Fellowship, NIAMS, NIH

Postgraduate Experience:

1982-83	Medical Internship, The Faulkner Hospital, Boston, MA
1983-89	Medical and Senior Staff Fellow, National Institutes of Health, Bethesda, MD
1989-91	Medical Officer, Center for Biologics Evaluation and Research, FDA, Bethesda, MD
1991-93	Tenured Senior Investigator, Division of Viral Products, CBER/FDA, Bethesda, MD.
1993 - 2007	Chief, Retroviral Immunology Section, Division of Viral Products, CBER/FDA, Bethesda, MD.
2007 -	Chief, Immune Modulation Section, Laboratory of Experimental Immunology, NCI/NIH, Frederick, MD.

Grants: (since 2000)

Interagency Agreement, NIAID	\$ 25,000, 2000
Research Grant, USAMRIID	\$ 202,000, 2000
National Vaccine Program,	\$ 79,000, 2000
CBER Counter-BioT Program	\$ 434,000, 2000

Patent income	\$ 175,000, 2000
CRADA - Coley Pharm	\$ 250,000, 2001
National Vaccine Program,	\$ 76,000, 2001
Research Grant, USAMRAA	\$ 120,000, 2002
CBER Counter-BioT Program	\$ 92,000, 2002
CRADA - Coley Pharm	\$ 250,000, 2002
Patent income	\$ 95,000, 2002
CBER Counter-BioT Program	\$ 40,000, 2003
Research Grant, USAMRAA	\$ 429,000, 2003
Research Grant, DARPA	\$ 257,000, 2003
Patent income	\$ 143,000, 2003
National Vaccine Program	\$ 79,000, 2004
Patent income	\$ 87,000, 2004
National Vaccine Program	\$ 223,000, 2005
Patent income	\$1,050,000, 2005
Defense Threat Reduction Agency	\$ 225,000, 2006
National Vaccine Program	\$ 180,000, 2006
Defense Threat Reduction Agency	\$ 225,000, 2007
Defense Threat Reduction Agency	\$ 215,000, 2008

Honors:

University Scholar, St. Joseph's University, 1976
 Presidential Scholar, St. Joseph's University, 1972 - 1976
 Summa Cum Laude, St. Joseph's University, 1976
 Medical Scientist Training Program, Univ of Penna, 1976 - 1982
 Baldwin Lucke Prize in Experimental Medicine, 1981
 R.G. Williams Prize in Basic Medical Science, 1982
 Regional Rheumatology Award, Amer. Rheumatology Association, 1987
 Senior Rheumatology Award, ARA, 1987
 Outstanding Achievement Award in the Biological Sciences, Washington Academy of Science, 1988
 Public Health Service Outstanding Service Medal, 1992
 Member, American Society for Clinical Investigation, 1992
 Public Health Service Outstanding Unit Citation, 1994
 Kunkel Award for Outstanding Research in the field of Rheumatology, American College of Rheumatology, 1994
 Outstanding Scientist Award, Center for Biologics Evaluation and Research, FDA 1995
 Outstanding Scientist of the Year Award, Food and Drug Administration, 1995
 Public Health Service Meritorious Service Medal, 1997
 Arthritis Foundation "Hero", 2001
 Public Health Service Distinguished Service Medal, 2004

Counter-Bioterrorism Activities

Security Clearance: **Secret**

Chairman, CBER Bioterrorism Response Working Group, 1998 - 2002.
 Organizer, CBER-USAMRIID Symposium on Bioterrorism Threats, Oct 1999.
 Participant, AMEDD Technology Workshop, Department of the Army, McLean, VA 1999.
 Speaker, "Current capabilities of biowarfare pathogens", SAG 2015 Threat Assessment Conference, National Intelligence Office, Mclean, VA 2000.
 Speaker, "Use of CpG Oligonucleotides to prevent infection by biowarfare agents", USAMRIID, Ft. Detrick, MD, 2000
 Participant, Biological Terrorism and Technology-Based Threat Assessment Workshop, Institute for Security Technology Studies, Dartmouth College, NH, 2000.
 Participant, Future Technology Seminar 2000, US Army Training and Doctrine Command, Ft. Monroe, VA, 2000.
 Speaker, "Induction of protective immune responses by CpG oligodeoxynucleotides", Biodefense: Research, Technologies and Applications, McLean, VA 2002.
 Participant, Biosecurity 2002 Conference, Las Vegas, NV 2002.
 Participant, Unconventional Pathogen Countermeasures conference, Galveston, Tx, 2003
 Member, Scientific Advisory Committee, Agile Vaccine Program, WRAIR
 Lecture, "Use of CpG Oligonucleotides for Biodefense", University of Texas Medical Branch, Galveston, Tx, 2003
 Member, Information Panel on Advances in Biological Threat Reduction, Miter Corporation, Vienna, VA, 2004
 FDA representative, Molecular Vaccine Interagency Working Group, White House Conference Center, 2004
 Speaker, "Use of CpG DNA and other TLR Ligands to Modulate Host Defense", American Academy of Allergy, Asthma and Immunology Annual Meeting, San Francisco, CA, 2004
 Speaker, "The Application of Immunotherapies to Biodefense", Biodefense Vaccines, Therapeutics and Diagnostics, Washington DC, 2004.
 Speaker, "The Use of CpG Oligonucleotides to Modulate Host Defense", First National Congress on Public Health Readiness, Washington DC, 2004.

Scientific Reviewer for the following journals:

AIDS
 American Journal of Pathology
 Arthritis and Rheumatism
 Blood
 Cancer Research
 Clinical Immunology and Immunopathology
 Human Gene Therapy

Immunopharmacology
Immunity
Journal of Acquired Immune Deficiency Syndrome
Journal of Clinical Immunology
Journal of Clinical Investigation
Journal of Experimental Medicine
Journal of Immunology
Journal of Immunological Methods
Journal of Molecular Immunology
Journal of Rheumatology
Journal of Virology
Nature Biotechnology
Proceedings of the National Academy of Sciences, USA
Scandinavian Journal of Immunology
Vaccine
Viral Immunology

Scientific Advisor or Reviewer for the following organizations:

American Rheumatology Association: Grants Review
American College of Rheumatology: Abstract Selection
Arthritis Council of Great Britain
Arthritis Council of Canada
Booz Allen and Hamilton
Broad Foundation
Defense Sciences Research Council, Universal Medical Countermeasures
Defense Threat Reduction Agency
Dept. of Veterans Affairs - Career Development Grants
Hughes Medical Research Institute
National Intelligence Office, Science and Technology Expert

Partnership (STEP)
NIAID: Extramural Program Project Grants Review
NIAID Malaria Vaccine Initiative
NCI: Extramural Program Project Grants Review
Office of Woman's Health
Sabin Vaccine Institute
Scientific Advisory Committee, Agile Vaccine Program, WRAIR
United States - Israel Binational Science Foundation

Teaching Appointments:

Lecturer, Foundation for Advanced Education in the Sciences, NIH. Course
Title: "Seminars in Immunology" (since 1991).
Lecturer, FAES, NIH. Course Title: "Cellular and Molecular
Mechanisms of Immunity".
Lecturer, Center for Drug Evaluation and Research Staff College,

FDA. Course Title: "Applied Immunology" (Since 1993).

Membership in Professional Societies:

American Association of Immunologists
American Association for the Advancement of Science
American Society for Clinical Investigation
New York Academy of Sciences

Selected Professional Presentations (from over 200 presentations)

Speaker, Conference on Immune Intervention, Montpellier, France, 1986.
Speaker, "B cell activation in Autoimmune Disease", Yokohama City University, Japan, 1988.
Speaker, "B cell activation in SLE", Combined Clinical Conference on Systemic Lupus Erythematosus, (Grand Rounds) NIH, 1991.
Chairman, Session on Murine Models of SLE, UCLA Symposium on Self Reactivity and its Regulation, Keystone, CO., 1991.
Speaker, "Cell line issues related to specific expression vectors", Fifth ABC International Biotechnology Meeting, Washington, DC. 1991.
Speaker, "Active and passive immunotherapy of HIV", VII International Conference on AIDS, Amsterdam, The Netherlands, 1992
Speaker, "Polyclonal B cell activation in Lupus", International Symposium on Immunobiology of Proteins and Peptides, Alberta, Canada, 1992.
Speaker, "Abnormalities in Lymphokine Production in HIV-Infected Patients", Yokohama City Medical School, Yokohama, Japan, 1994.
Speaker, "Alterations in the ratio of Th1 : Th2 type cytokine secreting cells in Pathologic States", Shimane Medical Univ., Shimane, Japan, 1994.
Chairman "Immunogenicity: Intended and Unintended", FDA Intramural Immunology Workshop, Rockville, MD, 1995
Co-Chair, "Immunity Induced by DNA Vaccines" American Society for Microbiology, New Orleans, LA, 1996.
Speaker, "The Scientific Future of Genetic Immunizations", American Academy of Microbiology, Aruba, Dutch Antilles, 1996.
Speaker, "DNase: Novel Therapy for the treatment of SLE", American College of Rheumatology National Meeting, Orlando, FL, 1996.
Speaker, "Contribution of CpG motifs to the immunogenicity of DNA vaccines", Mt. Sinai Medical Center, New York, NY, 1997.
Speaker, "Tolerance Induction by DNA Vaccines", Gordon Conference on Genetic Vaccination, Plymouth, New Hampshire, 1997.
Speaker, "Safety of DNA Vaccines", NIBSC, Hertfordshire, England, 1997.
Speaker, "Immunotherapeutic Potential of CpG Motifs", International Working Group on DNA Vaccines, London, England, 1998.
Chairman, Session on the "Mechanism of Action of DNA Vaccines", Keystone Symposium on DNA Vaccines, Snowbird, Utah, 1999

Speaker, "DNA Vaccines and Autoimmunity", 5th International Symposium on Autoimmunity, Limmisoli, Cyprus, 1999.

Speaker, "CpG motifs stimulate human cells", 1st International Symposium on CpG Oligonucleotides, Schloss Elmau, Germany, 1999.

Speaker, "Safety of DNA Vaccines", International Working Group on DNA Vaccines, Munich, Germany, 1999.

Speaker and Co-Chairman, "Cellular Uptake and Activation by CpG Oligonucleotides" Fourth NIH Symposium on Therapeutic Oligonucleotides, Bethesda, MD, 1999.

Speaker, "Immunogenicity of DNA vaccines", Millennium Conference on Nucleic Acid Therapeutics, Clearwater Beach, FL, 2000.

Speaker, "Capacity of DNA Vaccines to Induce Autoimmunity", WHO International Workshop on Nucleic Acid Vaccines, Geneva, Switzerland, 2000.

Speaker, "Therapeutic Applications of CpG Oligonucleotides", Yokohama City University Medical School, Yokohama, Japan, 2000.

Speaker, "Effect of Bacterial DNA on the Development of Autoimmune Disease", Japanese International Rheumatology Meeting, Yokohama, Japan, 2000.

Speaker, "Activity of Immunostimulatory and Immunosuppressive Motifs in Mammalian and Bacterial DNA", Japanese National Institutes of Health, Tokyo, Japan, 2000.

Speaker, "Safety of DNA Vaccines", Euregenethy Forum on Gene Therapy, Paris, France, 2000.

Speaker, "Immunostimulatory and immunosuppressive activity of DNA", LFKRI, New York, NY, 2001.

Speaker, "Immunoprotective activity of CpG Oligonucleotides", 2nd Annual Symposium on the Immunology of Infectious Disease, USUHS, Bethesda, MD, 2001.

Speaker, "CpG ODN induce protection against biothreat pathogens", 2nd International Symposium on CpG Oligos, Amelia Island, FL, 2001.

Speaker, "CpG adjuvant activity", Vaccines of the future: from rational design to clinical development. Paris, France, 2001.

Speaker, "Role of CpG stimulatory and inhibitory motifs in innate and cognate immune responses", Keystone Symposium, Breckenridge, CO. 2002.

Speaker, "CpG Oligonucleotides as Immune Adjuvants", IMV 2002 Vaccine Conference, Prague, Czechoslovakia

Co-chairman and speaker, "Innate immunity Session", Trudeau Institute Symposium on Immunity to Viral Infections, Saranac Lake, NY, 2002.

Speaker, "Induction of protective immune responses by CpG oligodeoxynucleotides", Biodefense: Research, Technologies and Applications, McLean, VA 2002.

Speaker, "Suppressive Oligonucleotides regulate CpG Induced Immune

Activation", 6th NIH Symposium on Therapeutic Oligonucleotides. Bethesda, MD 2002.

Chairman, Workshop on "Evaluating Nucleic Acid Based Vaccines", 7th Symposium on Regulatory and Analytical Sciences, San Francisco, CA 2003.

Plenary Lecture, "Science Based Regulation of DNA Vaccines", 7th Symposium on Regulatory and Analytical Sciences, San Francisco, CA 2003.

Lecture, "Use of CpG Oligonucleotides for Biodefense", University of Texas Medical Branch, Galveston, Tx, 2003

Speaker, "Protective Innate Immune Responses Elicited by CpG Nucleotides", FDA Science Forum, Washington, DC, 2003

Speaker, "Harnessing Immunostimulation CpG Oligonucleotides for BioDefense", The Emerging Role of Toll-like Receptors in Biology and Medicine, Boston University School of Medicine, Boston MA, 2003.

Speaker, "Immunomodulatory Activity of DNA", Keystone Symposium on Vaccines, Keystone, CO, 2004.

Speaker, "Use of CpG DNA and other TLR Ligands to Modulate Host Defense", American Academy of Allergy, Asthma and Immunology Annual Meeting, San Francisco, CA, 2004.

Lecture, "Therapeutic Applications of Immunomodulatory Oligonucleotides", Elkin Lecture Series, Winship Cancer Institute, Emory University, Atlanta, GA, 2004.

Lectures "Mode of Action of Immune Adjuvants" and "Safety of CpG-based Immune Adjuvants", World Health Organization Global Vaccine Safety Advisory Committee, Geneva, Switzerland, 2004.

Speaker "How CpG Adjuvants Interface with the Innate Immune System", Conference on Innate and Adaptive Immunity, Annecy, France, 2004.

Speaker, "The Use of CpG Oligonucleotides to Modulate Host Defense", First National Congress on Public Health Readiness, Washington DC, 2004.

Speaker, "Use of CpG Oligonucleotides as Vaccine Adjuvants", NIH Vaccine Working Group, Bethesda, MD 2005

Speaker, "Modulation of Disease Susceptibility through Activation and Suppression of the Innate Immune System", Society of Toxicological Pathology Annual Meeting, Washington DC, 2005.

Speaker, "Enhancement of Anthrax Vaccination in Primates by CpG ODN", Oligonucleotide Therapeutics Society Meeting, New York, NY, 2005.

Chairman, Session on "Innate Immunity and Antigen Presentation", NIH Immunology Retreat, Airlie, VA, 2005.

Speaker, "Innate Immune Modifiers: Activity and Safety as Vaccine Adjuvants", Medical Countermeasures and Innate Immunity, National Defense University, Washington DC, 2005.

Speaker, "Proposed Guidance on Prophylactic DNA Vaccines", 3rd International Symposium on DNA Vaccines, London, England, 2006.

Speaker, "Use of CpG oligonucleotides to improve host protection against infectious pathogens", Keystone Symposium on Viral Immunity, Steamboat Springs, CO, 2006.

Speaker, "CpG oligonucleotides: role in vaccine development", 9th Annual conference on Vaccine Research, Baltimore, MD, 2006.

Speaker, "Therapeutic applications of immunomodulatory oligonucleotides", Johns Hopkins University, Baltimore, MD, 2006.

Moderator, Session on "DNA Vaccine Electroporation" and Speaker "Insights into FDA regulation of DNA vaccines", DNA Vaccines 2007, Malaga, Spain, 2007.

Speaker, "Gene Activation Cascade Mediated by CpG Oligonucleotides", Microbiology Technical University of Munich, Munich, Germany, 2007.

Speaker, "Immunomodulatory Oligonucleotides: Impact on Immunity and Disease", Immunology Interest Group, NIH, Bethesda, MD, 2007.

Speaker, "CpG Oligonucleotides as Adjuvants for the Anthrax Vaccine", Systems Integration in Biodefense, Washington, DC, 2007.

Co-organizer and speaker, "Impact of CpG and Suppressive Oligonucleotides on Diseases associated with Inflammation", Cancer and Inflammation Symposium, Washington, DC, 2007.

Co-organizer and symposium chairman, Molecular Vaccine Development Symposium, Islamorada, FL, 2008.

Chairman and speaker, "Factors influencing DNA vaccine development", DNA Vaccines Forum 2008, London, England, 2008.

Speaker, "Advice to young investigators", NCI Fellows Retreat, Ocean City, MD, 2008.

Speaker, "Enhancement of AVA immunogenicity by CpG Oligonucleotides", 11th Annual conference on Vaccine research, Baltimore, MD, 2008.

Speaker, "Immunomodulatory activity of CpG ODN: opportunities for TLR based immune therapy to treat infectious diseases and cancer", Bilkent University, Ankara, Turkey, 2008 .

Speaker, "Immunosuppressive DNA to treat systemic inflammatory and autoimmune disease", GATA Medical School, Ankara, Turkey, 2008.

Patents (selected from over 150 patents in the area of CpG and suppressive ODN)
 Immunostimulatory Nucleic Acid Molecules. Patent No. 6,207,646 B1.
 Use of CpG Oligodeoxynucleotides to Encourage Angiogenesis
 DHHS Reference No. E-328-01/0.
 Immune Activation by Double-Stranded Polynucleotides. Application No.
 09/151,612
 Identification of DNA Sequence Motifs that Suppress the Immune Response to
 CpG DNA DHHS Reference No. E-218-01/0.
 Use of Sterically Stabilized Cationic Liposomes to Efficiently Deliver CpG
 Oligonucleotides in vivo DHHS Reference No. E-215-01/0.
 Novel Method for Rapidly Generating Mature Dendritic Cells from Peripheral
 Blood Monocytes and Myeloid Precursors DHHS Reference No. E-214-
 01/0
 Method of Treating and Preventing Infections in Immunocompromised Subjects
 with Immunostimulatory CpG Oligonucleotides DHHS Reference No. E-
 153-02/0.
 Methods for Treating and Preventing Infectious Disease Serial number
 10/187,489.
 Methods for Treating and Preventing Infectious Disease in Non-human Animals.
 Serial number 09/629,477.
 Methods and Products for Treating HIV Infection. Serial No. 09/931,583.
 Oligodeoxynucleotide and its use to Induce and Immune Response. DHHS
 Reference No. E-147-99/2
 Method for Treating Inflammatory Arthropathies with Suppressors of CpG
 Oligonucleotides. DHHS Reference No. E-154-02/2.
 Multiple CpG Oligodeoxynucleotides and their Use to Induce and Immune
 Response. DHHS Reference No. E-078-00/1.
 Immunostimulatory RNA/DNA hybrid molecules. Pub #: US-2004-0052763-A1

BIBLIOGRAPHY

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- 2) **Klinman DM** and McKearn TJ: Dialyzable serum components support the growth of hybridoma cell lines *in vitro*. J. Immunol. Meth. 42:1, 1981.
- 3) **Klinman DM**, Smilek DE and McKearn TJ: Class I major histocompatibility gene products of the Brown Norway rat display two major antigenic regions. J. Immunol. 129:1204, 1982.
- 4) **Klinman DM**: Immune recognition of Class I Major Histocompatibility Antigens. Thesis. 1982.
- 5) **Klinman DM**: Analysis of non-dominant idiotypes during alloimmune responses. Cell. Immunol. 87:126, 1984.
- 6) Miller ML, Raveche ES, Laskin CA, **Klinman DM** and Steinberg AD: Genetic studies in NZB mice. VI. Association of autoimmune traits in recombinant inbred lines. J. Immunol. 133:1325, 1984.
- 7) Mountz JD, Mushinski JF, **Klinman DM** and Steinberg AD: Autoimmunity and increased c-myc transcription. Science 226:1087, 1984.
- 8) Hudgins CC, Steinberg RT, **Klinman DM**, Reeves JP and Steinberg AD: Studies on consomic mice bearing the Y chromosome of the BXSB mouse. J. Immunol. 134:3849, 1985.
- 9) Smith HR, Yaffe LJ, Chused TM, Raveche ES, **Klinman DM** and Steinberg AD: Analysis of B cell subpopulations: I. Relationships among splenic χ id, Ly 1⁺ and LyB 5⁺ cells. Cell. Immunol. 92:190, 1985.
- 10) Steinberg AD, Triem KH, Smith HR, Laskin CA, Rosenberg YJ, **Klinman DM**, Mushinski JF and Mountz JD: Studies of the effects of Y chromosome factors on the expression of autoimmune disease. Ann. NY Acad Sci. 1985.
- 11) Mountz JD, Smith HR, **Klinman DM**, Mushinski JF and Steinberg AD: Modulation of c-myc oncogene transcription in autoimmune disease by cyclophosphamide. J. Immunol. 135:2417, 1985.
- 12) Steinberg AD, **Klinman DM**, Rosenberg YJ, Honda M, Santoro TJ, Reeve JP, Seldin MF, Mushinski JF and Mountz JD: Autoimmune lymphoproliferative disorders: Studies of pathogenesis and approaches to therapy. In: Mediators of Immune Regulation and Immunotherapy, Ed. Singhal SK. Elsevier Science Publishing Co., Inc., London, Canada, p. 213, 1985.
- 13) **Klinman DM** and Steinberg, AD: Autoimmunity, In: Systemic Lupus Erythematosus Textbook. Ed: Lahita RG. John Wiley Co., NY p. 1 1986.
- 14) **Klinman DM**, Lefkowitz MD, Barrett R, Honda M and Steinberg AD: Suppression of autoantibody production with anti-class II antibodies. In: Immune Intervention, Vol 2 Eds. Brochier J, Clot J and Sany J. Academic Press, NY. p. 87, 1986.
- 15) **Klinman DM**, Lefkowitz MD, Raveche ES, Honda M and Steinberg AD: Effect of anti-Ia treatment on the production of anti-DNA antibody by NZB mice. Eur. J. Immunol. 16:939, 1986.
- 16) **Klinman DM** and Steinberg AD: Idiotype and autoimmunity. Arthritis Rheum. 29:697, 1986.
- 17) **Klinman DM**, and Steinberg AD: Proliferation of anti-DNA producing NZB B cell in a non-autoimmune environment. J. Immunol. 137:69, 1986.
- 18) **Klinman DM**, Mushinski JF, Honda M, Ishigatsubo Y, Mountz JD, Raveche ES and

- Steinberg AD: Oncogene expression in autoimmune and normal peripheral blood mononuclear cells. J Exp. Med. 163:1292, 1986.
- 19) **Klinman DM**, Steinberg AD and Mushinski JF: Oncogene expression in angioimmunoblastic lymphadenopathy: Effect of cyclophosphamide therapy. Lancet 2:1055, 1986.
 - 20) Steinberg AD, **Klinman DM**, Kastner DL, Seldin MF, Gause WC, Scribner CL, Britten JL, Siegel JM and Mountz JD: Genetic and molecular genetic studies of murine and human lupus. Proceedings of the First International Symposium on SLE. J. Rheumatol. (Sup) 13, 14:166, 1987.
 - 21) **Klinman DM** and Cohen DI: Preserving primary cDNA libraries: Analyt. Biochem. 161:85, 1987.
 - 22) Scribner CL, Hansen CT, **Klinman DM** and Steinberg AD: The interaction of the xid and me genes. J. Immunol. 138:3611, 1987.
 - 23) **Klinman DM** and Steinberg AD: Systemic autoimmune diseases arise from polyclonal B cell activation. J. Exp. Med. 165:1755, 1987.
 - 24) Mock B, Skurla R, Huppi K, D'Hoostelaere L, **Klinman DM** and Mushinski JF: A restriction fragment length polymorphism at the murine c-myb locus. Nuc. Acids Res. 15:4700, 1987.
 - 25) **Klinman DM** and Steinberg AD: Preferential in vivo expansion of auto-antibody secreting B cells derived from normal DBA/2 mice. J. Immunol. 139:2284, 1987.
 - 26) Ishigatsubo Y, **Klinman DM** and Steinberg AD: Transfer of anti-DNA producing B cells from NZB to unmanipulated xid recipients. Clin. Immunol. and Immunopathol. 45:244, 1987.
 - 27) **Klinman DM** and Steinberg AD: Novel ELISA and ELISA spot assays used to quantitate B cells and serum antibodies specific for T cell and bromelated mouse red blood cell autoantigens. J. Immunol. Meth. 102:157, 1987.
 - 28) Siegel J, Turner A, **Klinman DM**, Wilkinson M, Steinberg AD, MacLeod C, Paul WE, Davis M and Cohen DI: Sequence analysis of an X-linked lymphocyte-regulated gene family (XLR): homologies with the nuclear structural proteins, Lamins A and C. J. Exp. Med. 166:1702, 1987.
 - 29) Steinberg AD, Seldin MF, Jaffe ES, Smith HR, **Klinman DM**, Krieg AM, and Cossman J. Angioimmunoblastic Lymphadenopathy with Dysproteinemia. Annals Int. Med. 108:575, 1988.
 - 30) Steinberg AD and **Klinman DM**: Pathogenesis of Systemic Lupus Erythematosus. In: Rheumatic Disease Clinics of North America. Ed: Klippel JH. W.B. Saunders, Co. Phila, Pa. pp. 25-42. 1988.
 - 31) Jensen L, Kuff EL, Wilson SH, Steinberg AD and **Klinman DM**: Antibodies from patients and mice with autoimmune disease react with recombinant HNRNP core protein A1. J. Autoimmunity 1:73, 1988.
 - 32) **Klinman DM**, Ishigatsubo Y and Steinberg AD: Acquisition and maturation of expressed B cell repertoires in normal and autoimmune mice. J. Immunol. 141:801, 1988.
 - 33) **Klinman DM**, Ishigatsubo Y and Steinberg AD: Pathogenesis of generalized autoimmunity: In vivo studies of the interaction between B cells and their internal milieu. Cell. Immunol. 17:360, 1988.
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Vaccination with Novel Immunostimulatory Adjuvants against Blood-Stage Malaria in Mice

Zhong Su,¹ Mi-Fong Tam,¹ Dragana Jankovic,² and Mary M. Stevenson^{1*}

Centre for the Study of Host Resistance, McGill University Health Centre Research Institute, McGill University, Montreal, Quebec, Canada,¹ and Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland²

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An important aspect of malaria vaccine development is the identification of an appropriate adjuvant which is both capable of stimulating a protective immune response and safe for use by humans. Here, we investigated the feasibility of using novel immunostimulatory molecules as adjuvants combined with a crude antigen preparation and coadsorbed to aluminum hydroxide (alum) as a vaccine against blood-stage *Plasmodium chabaudi* AS malaria. Prior to challenge infection, immunization of genetically susceptible A/J mice with the combination of malaria antigen plus recombinant interleukin-12 (IL-12) in alum induced a Th1 immune response with production of high levels of gamma interferon (IFN- γ) and diminished IL-4 levels by spleen cells stimulated in vitro with parasite antigen compared to mice immunized with antigen alone, antigen in alum, or antigen plus IL-12. Mice immunized with malaria antigen plus recombinant IL-12 in alum had high levels of total malaria-specific antibody and immunoglobulin G2a. Compared to unimmunized mice, immunization with antigen plus IL-12 in alum induced the highest level of protective immunity against challenge infection with *P. chabaudi* AS, which was evident as a significantly decreased peak parasitemia level and 100% survival. Protective immunity was dependent on CD4⁺ T cells, IFN- γ , and B cells and was long-lasting. Replacement of IL-12 as an adjuvant by synthetic oligodeoxynucleotides (ODN) containing CpG motifs induced a similar level of vaccine-induced protection against challenge infection with *P. chabaudi* AS. These results illustrate that it is possible to enhance the potency of a crude malaria antigen preparation delivered in alum by inclusion of immunostimulatory molecules, such as IL-12 or CpG-ODN.

Malaria vaccine development efforts during the past 20 years have been aimed at antigen identification, gene cloning, and expression of recombinant molecules (17). This has resulted in a number of promising blood-stage-derived recombinant antigens for inclusion in subunit vaccines, including MSP1, MSP2, MSP3, MSP4, MSP5, AMA1, PfEMP1, RESA, RAP1, and RAP2 (10, 18). Clinical trials with many of these candidates have been conducted or are ongoing (10, 13, 18). It is anticipated that the success of these trials may potentially lead to a vaccine capable of saving millions of lives each year from malaria.

An important aspect of vaccine development against infectious diseases, including malaria, is the identification of an appropriate adjuvant which is both capable of stimulating a protective immune response and safe for use by humans. Aluminum hydroxide, usually referred to as alum, which is approved as an adjuvant for use by humans, is not always the most appropriate adjuvant, given its potential to stimulate a Th2 type immune response characterized by immunoglobulin G1 (IgG1) and IgE production and the lack of induction of cytotoxic T-cell responses (5). This is particularly problematic in the development of vaccines against diseases caused by intracellular pathogens such as protozoan parasites, including intraerythrocytic *Plasmodium* parasites, the causative agent of

malaria. Protective immunity against intracellular pathogens is generally dependent on Th1 type immune responses. However, protective immunity against blood-stage malaria is particularly complex and requires a concerted effort by a Th1 type cellular immune response and humoral immunity possibly involving a Th2 type response (24, 29). Rodent studies have revealed a role for CD4⁺ T cells, B cells, and antibody in mediating naturally induced immunity against primary blood-stage infection (24, 29). The importance of CD4⁺ T cells in immunity induced by vaccination with a defined antigen, such as MSP1, is less clear, although high titers of antigen-specific antibody correlate with protective immunity induced in mice by vaccination with the 19-kDa carboxyl-terminal fragment of MSP1 derived from *Plasmodium yoelii* (reviewed in reference 13).

Interleukin-12 (IL-12) plays an essential role in the differentiation of CD4⁺ lymphocytes by promoting Th1 while suppressing Th2 cell development, thereby favoring gamma interferon (IFN- γ) production and elevated IgG2a levels (31). Because of its potent immunoregulatory properties, this cytokine has been used successfully as a vaccine adjuvant in models of intracellular infections, such as *Leishmania major* (1, 21) and *Listeria monocytogenes* (27), which require induction of Th1 responses for protective immunity. Consistent with the ability of IL-12 to promote a Th1-dependent immune response and to dampen a Th2 response, Wynn and colleagues (46–48) demonstrated that, as an adjuvant, IL-12 not only influences the promotion of protective Th1-dependent vaccine-induced immunity against *Schistosoma mansoni* but also prevents Th2-dependent pathology associated with this helminth parasite.

* Corresponding author. Mailing address: Montreal General Hospital Research Institute, MUHC RI, Room L11-409, 1650 Cedar Ave., Montreal, Quebec H3G 1A4, Canada. Phone: (514) 934-1934, ext. 44507. Fax: (514) 934-8332. E-mail: mary.m.stevenson@mcgill.ca.

Furthermore, as a vaccine adjuvant, IL-12 enhances both cell-mediated immune responses and augments antigen-specific IgG1, IgG2a, and IgG3 antibody levels, especially when the antigen and IL-12 are simultaneously coadsorbed to alum (19, 48).

In previous studies, our laboratory demonstrated that treatment of genetically susceptible A/J mice with exogenous IL-12 during primary *Plasmodium chabaudi* AS infection induces protective type 1 immunity, resulting in a less severe course of infection and survival (37). Studies with IL-12 p40-deficient mice confirmed the key role of IL-12 in inducing protective Th1 responses involving IFN- γ during the acute phase of infection and demonstrated that IL-12 is also important for antibody-mediated immunity during the chronic stage and in a challenge infection (39).

In the present study, we investigated the potential of using IL-12 as an adjuvant in a vaccine against blood-stage *P. chabaudi* AS malaria in A/J mice. Since our focus was to define the efficacy and characterize the protective immune mechanisms induced by the combination of an immunostimulatory molecule with malaria antigen, we used murine recombinant IL-12 together with a crude preparation of whole antigen from *P. chabaudi* AS parasitized red blood cells (PRBC). With this combination coadsorbed to alum, we report the induction of a protective antimalarial immune response in susceptible A/J mice characterized by the production of high levels of IFN- γ and parasite-specific IgG2a and protection against challenge infection with blood-stage *P. chabaudi* AS. Protective immunity was found to be dependent on CD4⁺ T cells, B cells, and IFN- γ and to be long-lasting. Replacement of IL-12 as an adjuvant by synthetic oligodeoxynucleotides (ODN) containing CpG motifs was found to induce a similar level of vaccine-induced protection against challenge infection with *P. chabaudi* AS.

MATERIALS AND METHODS

Mice. Age- and sex-matched mice, 6 to 8 weeks old, were used in all experiments. A/J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and C57BL/6 mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). IFN- γ knockout (GKO) mice on the C57BL/6 background were bred in the animal facility of the Montreal General Hospital Research Institute from breeding pairs of GKO mice, which were originally from Genentech, Inc. (South San Francisco, Calif.) and backcrossed onto the C57BL/6 strain for eight generations by F. P. Heinzel (Case Western Reserve University School of Medicine, Cleveland, Ohio) (16). B-cell-deficient μ -MT with targeted disruption of the membrane exon of the immunoglobulin μ -chain gene or B-cell knockout (BKO) mice were originally derived on a 129 \times C57BL/6 background and backcrossed to the C57BL/10 background for 12 generations (20, 22). B-cell-deficient μ -MT and wild-type C57BL/10SgSnAi mice were obtained from Taconic Farms, Inc. (Germantown, N.Y.).

CD4⁺ T-cell depletion. Monoclonal anti-CD4 antibody from the hybridoma clone GK1.5 was raised as ascites fluid in BALB/c mice as previously described (33). The ascites fluid was delipidified, dialyzed, and quantitated for the concentration of rat IgG. Mice were treated with the first dose of 500 μ g of anti-CD4 antibody intraperitoneally (i.p.) 3 days prior to infection. Following infection, 200 μ g was administered i.p. 3 times per week until the end of the experiment. Control mice received purified rat IgG at similar dosages and timing. Treatment with GK1.5 monoclonal antibody consistently depletes >98% of CD4⁺ T cells based on fluorocytometric analysis (33, 37) and functional studies (33).

***P. chabaudi* AS infection and antigen preparation.** *P. chabaudi* AS was maintained as previously described (33). Naive and immunized mice were infected i.p. with 10⁶ PRBC. The course and outcome of infection were monitored by previously described procedures (33). For the determination of cytokine and antibody levels in sera, mice were sacrificed at the indicated times and blood was obtained by cardiac puncture, allowed to clot for 30 min at 4°C, and centrifuged at 3,000 \times g for 3 min. Sera were collected and stored at 4°C for measurement of IL-12

p70 or at -20°C for determination of the levels of other cytokines and malaria-specific antibodies.

Antigen was prepared by modification of a freeze-thaw protocol described by Amante and Good (2). Briefly, blood from A/J mice with parasitemias of 40 to 45% was collected, pooled, and centrifuged at 300 \times g for 10 min. The red blood cell pellet was subjected to 2 rounds of lysis with distilled H₂O and centrifugation at 10,000 \times g for 25 min. After 2 washes with phosphate-buffered saline (PBS), the parasite pellet was resuspended in PBS and subjected to 3 cycles of freeze-thaw at -70°C and 37°C. The suspension, containing both soluble and particulate antigens, was further disrupted by passage 2 to 3 times through a syringe with a 25-gauge needle.

Immunization protocol. An amount of malaria antigen equivalent to 1 \times 10⁷ to 1.5 \times 10⁷ PRBC was mixed with 1 μ g of murine recombinant IL-12 (a kind gift from Wyeth Genetics Institute, Cambridge, Mass.) to a volume of 50 μ l with PBS. An equal volume of alum (Imject alum; Pierce Chemical Co., Rockford, Ill.) was added, and the suspension was mixed thoroughly. Mice were immunized subcutaneously (s.c.) with 0.1 ml on the nape of the neck. Other groups of mice were also immunized in a similar manner with the following vaccine combinations: antigen suspended in PBS, antigen admixed in alum, and antigen admixed with 1 μ g of murine recombinant IL-12 in PBS. Three weeks later, the antigen-treated groups were boosted with the same amount of antigen in 0.1 ml of PBS injected i.p. Mice were challenged i.p. with 10⁶ PRBC 2 weeks later.

CpG DNA. ODN containing CpG motifs (CpG-ODN no. 1826) and control ODN (no. 1982) were a kind gift of Coley Pharmaceuticals Canada (Ottawa, Ontario, Canada). One hundred micrograms of CpG-ODN or control ODN was admixed with antigen and alum and used according to the standard immunization protocol described above.

Spleen cell culture and proliferation assay. Spleens from immunized mice were removed aseptically and pressed through a sterile fine-wire mesh with 10 ml of RPMI 1640 (Gibco-Invitrogen, Burlington, Ontario, Canada) supplemented with 5% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah), 25 mM HEPES (Gibco-Invitrogen), 0.12% gentamicin (Schering, Montreal, Quebec, Canada), and 2 mM glutamine (Gibco-Invitrogen). Cell suspensions were centrifuged at 350 \times g for 10 min. Red blood cells were lysed with 0.175 M NH₄Cl, and the cells were washed twice in fresh medium. Membrane debris was removed by filtering the cell suspensions through sterile gauze. The viability of the cells was determined by trypan blue exclusion and was always >90%. Total cell counts were performed on individual samples. For proliferation assays, spleen cells were adjusted to 2.5 \times 10⁶ cells/ml and aliquots of 0.1 ml were plated in triplicate in 96-well flat-bottom plates, stimulated with 10⁶ washed PRBC/ml (as the malaria parasite antigen) or medium (as the control), and incubated for 72 h at 37°C in a humidified CO₂ incubator. During the last 16 h of culture, 1 μ Ci of [³H]thymidine (specific activity, 6.7 Ci/mmol) was added to each well, the cells were harvested with an automatic cell harvester, and the incorporated radioactivity was measured in a liquid scintillation counter. For determination of cytokine production, spleen cells were adjusted to 5 \times 10⁶ cells/ml and aliquots of 1 ml were plated in triplicate in 24-well tissue culture plates in the presence or absence of 10⁶ PRBC, as described above, and incubated for 48 h at 37°C in a humidified CO₂ incubator. Supernatants were collected, centrifuged at 350 \times g for 5 min, and stored at 4°C or at -20°C until assayed for cytokine levels.

Cytokine ELISAs. Cytokine levels in sera and spleen cell supernatants were measured by using two-site sandwich enzyme-linked immunosorbent assays (ELISAs) for IFN- γ and tumor necrosis factor alpha (TNF- α) as previously described (34, 37). For IL-4, the capturing and detecting antibodies were BVD4-1D11 monoclonal antibody (MAb) and biotinylated BVD6-24G2 MAb, respectively. For IL-10, JES5.2A5 MAb (American Type Culture Collection, Rockville, Md.) and biotinylated SXC-1 MAb (BD Bioscience, Mississauga, Ontario, Canada) were used as the capturing and detecting antibodies, respectively. Standard curves for each cytokine were generated by using recombinant cytokines (BD Bioscience). Reactivity was revealed by using 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate (Roche, Laval, Quebec, Canada) and optical density (OD) values were read in a microplate reader at 405 nm, with a reference wavelength of 492 nm.

Malaria-specific antibody ELISA. Serum levels of *P. chabaudi* AS-specific antibody isotypes were determined by ELISA. *P. chabaudi* AS antigen was prepared as described previously (49). Immulon II plates (Dynatech, Chantilly, Va.) were coated with parasite antigen at a concentration of approximately 4 to 5 μ g/ml in PBS based on an OD at 280 nm overnight at 4°C and subsequently blocked with 1% bovine serum albumin in PBS for 1 h. Individual serum samples were serially diluted twofold, and 50 μ l of each dilution was added to the plate and incubated for 2 h at room temperature. The data shown are based on values obtained at the following dilutions: total Ig, 1:20; IgG1, 1:10; IgG2a, 1:10. After extensive washing, horseradish peroxidase-conjugated goat anti-mouse isotype

TABLE 1. Antigen-specific spleen cell proliferation and cytokine responses in immunized mice prior to *P. chabaudi* AS challenge infection

Treatment group ^a	Antigen proliferation (cpm \pm SEM)	Level of cytokine (Mean \pm SEM):			
		IFN- γ (ng/ml)	TNF- α (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)
Untreated	526 \pm 71	0.39 \pm 0.39	98.41 \pm 10.22	254.27 \pm 52.80	0.76 \pm 0.01
Antigen	486 \pm 44	5.13 \pm 0.36	122.03 \pm 17.78	178.55 \pm 45.13	1.07 \pm 0.11
Antigen + alum	1,162 \pm 128	3.83 \pm 0.86	175.03 \pm 20.93	234.52 \pm 20.60	1.27 \pm 0.10
Antigen + IL-12	768 \pm 130	11.14 \pm 2.27	189.85 \pm 28.58	134.19 \pm 33.22	1.01 \pm 0.09
Antigen + IL-12 + alum	2,768 \pm 622 ^b	43.13 \pm 5.20 ^c	341.94 \pm 44.26 ^c	165.87 \pm 42.82 ^d	1.44 \pm 0.14 ^e

^a Groups of A/J mice (5 per group) were immunized s.c. with malaria antigen alone, antigen in alum, antigen plus 1.0 μ g of IL-12, or antigen plus 1.0 μ g of IL-12 in alum and boosted 3 weeks later by i.p. injection with antigen. Data from one of two replicate experiments are presented.

^b $P < 0.05$ for antigen plus alum versus antigen plus IL-12 plus alum.

^c $P < 0.0001$ for antigen plus alum versus antigen plus IL-12 plus alum.

^d $P < 0.01$ for antigen plus alum versus antigen plus IL-12 plus alum.

^e $P < 0.008$ for antigen plus alum versus antigen plus IL-12 plus alum.

antibodies (SBA, Birmingham, Ala.) were added and incubated at room temperature for another 2 h. Reactivity was visualized with ABTS substrate, and OD values were read in a microplate reader at 405 nm, with a reference wavelength of 492 nm. Antibody levels in serum are expressed as relative OD.

Statistical analysis. Data are presented as means \pm standard errors of the means. The statistical significance of differences in means between experimental and control groups was analyzed by Student's *t* test with SAS/STAT software (SAS Institute, Cary, N.C.). A *P* value of <0.05 was considered significant.

RESULTS

Immunization with malaria antigen plus IL-12 in alum induces a Th1 immune response. Since a strong Th1 immune response is associated with protective immunity to acute blood-stage *P. chabaudi* AS during a primary infection, the type of immune response induced by inclusion of IL-12 in a vaccine formulation was first evaluated. *P. chabaudi* AS susceptible A/J mice were immunized s.c. with a freeze-thaw preparation of blood-stage malaria antigen alone, antigen in alum, antigen plus IL-12, or antigen plus IL-12 in alum and boosted 3 weeks later by i.p. injection with antigen alone. Two weeks later, prior to challenge infection, immunized mice and untreated, control A/J mice were sacrificed and proliferation and cytokine production by spleen cells were analyzed in vitro. As shown in Table 1, immunization with either antigen in alum or the combination of antigen plus IL-12 in alum resulted in significantly increased antigen-specific proliferation compared to the response of control A/J mice ($P = 0.02$ and $P = 0.037$, respectively). However, the combination of antigen plus IL-12 in alum resulted in a greater than twofold increase in proliferation compared to antigen in alum, which represents a significant difference between the two groups. Furthermore, in comparison with spleen cells from mice immunized with antigen in alum, spleen cells from mice immunized with the combination of antigen plus IL-12 in alum produced significantly higher levels of the Th1 cytokines, IFN- γ and TNF- α , and significantly lower levels of IL-4. Spleen cells from mice immunized with the combination of antigen plus IL-12 in alum also produced modest levels of IL-10, which were significantly higher than the response of cells from mice immunized with antigen in alum.

The levels of total malaria-specific antibody and IgG1 and IgG2a in the sera of immunized A/J mice were also analyzed 2 weeks after boosting prior to challenge infection. Total malaria-specific antibody was significantly and similarly increased in the three groups of immunized animals compared with the levels of total specific antibody in mice immunized with antigen

alone (Fig. 1A). Malaria-specific IgG1 was significantly increased in the groups immunized with antigen in alum and the combination of antigen plus IL-12 in alum compared to IgG1 levels in mice immunized with antigen alone (Fig. 1B). However, the level of malaria-specific IgG1 was significantly higher in the group immunized with antigen in alum than in those immunized with the combination of antigen plus IL-12 in alum. The levels of specific IgG2a were significantly increased compared to controls only in mice immunized with the combination of antigen plus IL-12 in alum (Fig. 1C). These findings demonstrate that immunization with the combination of malaria antigen plus IL-12 in alum induced high levels of production of the Th1 cytokine IFN- γ and parasite-specific IgG2a. In addition, mice immunized with this combination produced significantly lower levels of antigen-specific IL-4 and IgG1 than did mice immunized with antigen in alum in the absence of IL-12.

Immunization with malaria antigen plus IL-12 in alum induces protection against challenge infection with blood-stage *P. chabaudi* AS. To compare the efficacy of vaccination with the various combinations in conferring protective immunity, groups of A/J mice, immunized as described above, were challenged i.p. with *P. chabaudi* AS 2 weeks after boosting and the course of parasitemia and the outcome of infection were monitored. Similar to control mice, mice immunized with antigen alone or antigen in alum suffered a severe course of parasitemia with high peak parasitemia levels and high mortality (Fig. 2A and C). Mice immunized with antigen plus IL-12 or antigen plus IL-12 in alum experienced less severe courses of infection with significantly lower peak parasitemia levels than control mice ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 2B). In the case of mice immunized with antigen plus IL-12 in alum, there was a delay of 1 to 2 days in peak parasitemia level compared to nonimmunized mice. Although antigen plus IL-12 was effective in significantly reducing peak parasitemia compared to control mice, only 60% (9 of 15) of mice immunized with this combination survived while 100% (25 of 25) of mice immunized with the combination of antigen plus IL-12 in alum survived challenge infection with *P. chabaudi* AS. These results indicate that antigen plus IL-12 in alum was the best combination for conferring protection against blood-stage malaria in terms of reduced parasitemia and enhanced survival.

Immunization with malaria antigen plus IL-12 in alum induces long-lasting protection. An important characteristic of

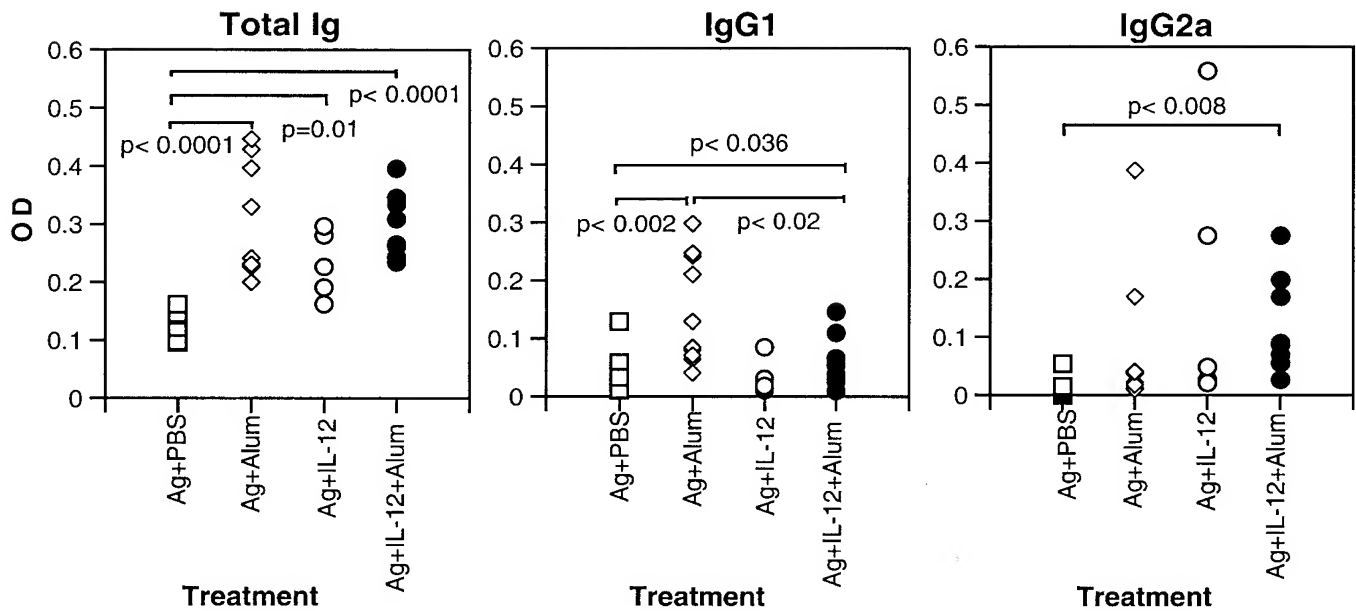


FIG. 1. Levels of malaria-specific antibodies in the sera of A/J mice immunized s.c. with antigen (Ag) alone, antigen in alum, antigen plus IL-12, or antigen plus IL-12 in alum and boosted 3 weeks later by i.p. injection with antigen. Two weeks later, sera were collected from immunized mice and the levels of total malaria-specific antibody, IgG1, and IgG2a were determined by ELISA. Data represent OD values for individual mice and are pooled from 2 experiments.

an effective malaria vaccine is that the elicited immunity is long-lasting. To address this issue, A/J mice were immunized with the combination of antigen plus IL-12 in alum and challenged as before, that is, 2 weeks after boosting, or 12 weeks after boosting. Similar to mice challenged 2 weeks after boosting, A/J mice challenged at 12 weeks were solidly immune (Table 2). Long-lasting protection induced in these animals by malaria antigen plus IL-12 in alum was evident by a number of parameters. Importantly, there was a significant decrease in peak parasitemia compared to nonimmunized A/J mice ($P < 0.001$). In addition, the number of days required to clear parasites from the blood of mice challenged 12 weeks after boosting was similar to that for mice challenged 2 weeks after boosting, and there was 100% survival among all immunized mice regardless of the time of challenge infection.

Protective immunity induced by immunization with malaria antigen plus IL-12 in alum requires CD4⁺ T cells and IFN- γ . To investigate the mechanism of protective immunity induced

by vaccination with the combination of antigen plus IL-12 in alum, immunized A/J mice were depleted of CD4⁺ T cells by treatment with GK1.5 MAb 3 days prior to and three times per week during the challenge infection with *P. chabaudi* AS. Parasitemia and survival were monitored for 4 weeks post-challenge infection. Consistent with the results shown above, intact immunized A/J mice suffered a mild course of infection and survived challenge infection. In contrast, CD4⁺-T-cell-depleted mice experienced fulminant infections with significantly higher peak parasitemia levels than rat IgG-treated mice ($P = 0.008$) (Fig. 3A), and the animals died by day 11 postchallenge.

To determine the role of IFN- γ in vaccine-induced protection, GKO mice on the resistant C57BL/6 background and wild-type C57BL/6 mice (36) were immunized with antigen plus IL-12 in alum. Immunized as well as untreated, control GKO and wild-type mice were challenged with *P. chabaudi* AS as described above. The course of parasitemia and outcome of infection were monitored for 4 weeks in control and immunized mice of both genotypes (Fig. 3B and C). As shown previously, control GKO mice developed significantly higher levels of peak parasitemia on day 7 than their wild-type counterparts (64.2 ± 3.35 versus 38.7 ± 4.43 , respectively; $P < 0.0001$). Furthermore, immunized wild-type C57BL/6 mice had a significantly lower peak parasitemia level which occurred 1 day later than that for wild-type mice without immunization ($P < 0.0001$) (Fig. 3B), indicating that immunization with antigen plus IL-12 in alum induced protection in resistant C57BL/6 as well as susceptible A/J hosts. In contrast to increased protection, as defined by the level of peak parasitemia, observed in wild-type mice, there was no significant difference in peak parasitemia levels in immunized versus untreated GKO mice (55.31 ± 1.37 versus 64.2 ± 3.35 , respectively; $P = 0.05$). The timing of the peak parasitemia was delayed from day 7 to day

TABLE 2. Long-term protection against blood-stage malaria induced by immunization with malaria antigen plus IL-12 in alum

Treatment group ^a (n)	% Peak parasitemia (mean \pm SEM)	Clearance by day:	% Survival
Untreated (10)	41.25 \pm 1.29		0
2 wk postboost (5)	15.80 \pm 2.29 ^b	14	100
12 wk postboost (5)	28.65 \pm 1.29 ^c	15	100

^a Groups of A/J mice ($n = 5$) were immunized s.c. with antigen plus 1.0 μ g of IL-12 admixed in alum and boosted 3 weeks later by i.p. injection with antigen. For each immunization group, age-matched untreated and immunized mice were infected i.p. with 10^6 PRBC at 2 or 12 weeks postboost. Since there were no significance differences in peak parasitemia or survival between the two untreated groups ($n = 10$), data have been pooled.

^b $P < 0.001$ compared to control.

^c $P < 0.001$ compared to control.

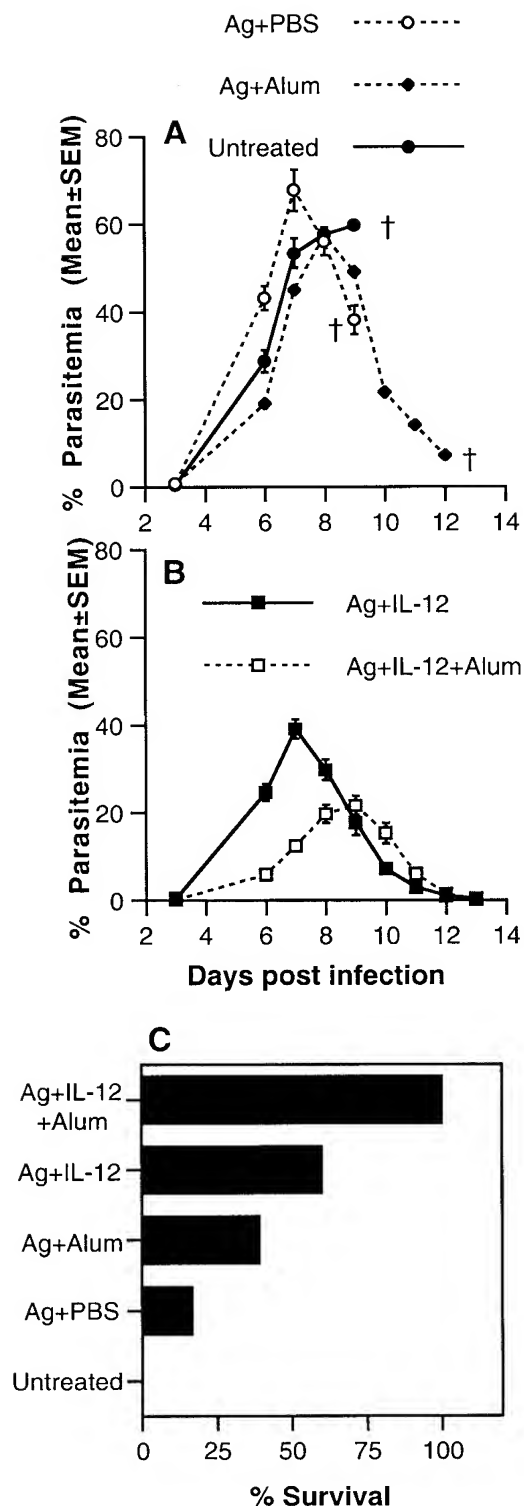


FIG. 2. Course of parasitemia and survival in A/J mice immunized s.c. with antigen (Ag) alone, antigen in alum, antigen plus IL-12, or antigen plus IL-12 in alum and boosted 3 weeks later by i.p. injection with antigen. Two weeks later, immunized and untreated control mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC. The percentage of PRBC in peripheral blood (A and B) was determined for each group of 5 mice. Data from one of two replicate experiments are presented. Mice were examined twice daily for the duration of the experiment for survival (C). Cumulative data from 6 experiments are presented.

9 in immunized mice compared to control GKO mice. However, 100% of GKO mice, whether immunized or not, succumbed to challenge infection by day 12 (40; data not shown). Taken together, these results demonstrate the crucial roles of $CD4^+$ T cells and IFN- γ in the development of protective immunity against blood-stage malaria induced by immunization with *P. chabaudi* AS antigen plus IL-12 coadsorbed to alum.

Protective immunity induced by immunization with malaria antigen plus IL-12 in alum requires B cells. As shown above, immunization of A/J mice with malaria antigen plus IL-12 in alum induced high levels of total malaria-specific antibody, IgG2a, and IgG1, and conferred the highest level of protection against challenge infection with blood-stage *P. chabaudi* AS. These observations suggested to us that the B-cell response is an integral component of the mechanism of protective immunity induced by immunization with the combination of malaria antigen and IL-12 coadsorbed to alum. The role of B cells in protective immunity induced by vaccination with antigen plus IL-12 in alum was further investigated by using B-cell-deficient μ -MT mice on the resistant C57BL/10 background (36). As previously observed (41, 43, 44), nonimmunized male (Fig. 4A) and female (Fig. 4C) B-cell-deficient mice compared to intact C57BL/10 mice (Fig. 4B and D) experienced recurrent bouts of recrudescence parasitemia until the experiment was terminated 90 days after challenge infection. Following immunization, peak parasitemia levels in male and female intact C57BL/10 mice were significantly decreased ($P < 0.001$ for male mice and $P < 0.05$ for female mice). Challenge infection was cleared in both male and female immunized C57BL/10 mice, although female mice experienced several recrudescence parasitemias between 5 and 10%. Despite immunization, male and female B-cell-deficient mice experienced peak parasitemias which were not significantly reduced compared to nonimmunized, B-cell-deficient mice. Although immunized B-cell-deficient mice suffered fewer and significantly lower recrudescence parasitemias than their nonimmunized counterparts, they were unable to clear the infection completely and low levels of parasitemia (1 to 5%) persisted throughout the chronic stage of infection until the experiment was terminated on day 90.

CpG-ODN can replace IL-12 as an adjuvant for immunization against blood-stage malaria. It is possible that other agents, such as CpG-ODN, with potent immunostimulatory properties could also be useful as an adjuvant in a vaccine against blood-stage malaria. CpG-ODN has been shown to induce production of IL-12, which, in turn, enhances IFN- γ production, antibody production by B cells, and cytotoxicity of NK cells and $CD8^+$ T cells (4, 5, 7, 15, 23). To determine whether CpG-ODN can replace IL-12 as an adjuvant in the blood-stage malaria vaccine, A/J mice were immunized with malaria antigen plus 100 μ g of CpG-ODN or control ODN in alum, by using the standard protocol, and challenged with *P. chabaudi* AS. As shown in Fig. 5, CpG-ODN was as effective as IL-12 in inducing protection against challenge infection with *P. chabaudi* AS. Mice immunized with malaria antigen plus CpG-ODN in alum had a course of parasitemia and 100% survival following challenge infection with *P. chabaudi* AS, which was similar to mice immunized with antigen plus IL-12 in alum. There was a significant decrease in the peak parasitemia levels of mice immunized with antigen plus CpG-ODN in alum com-

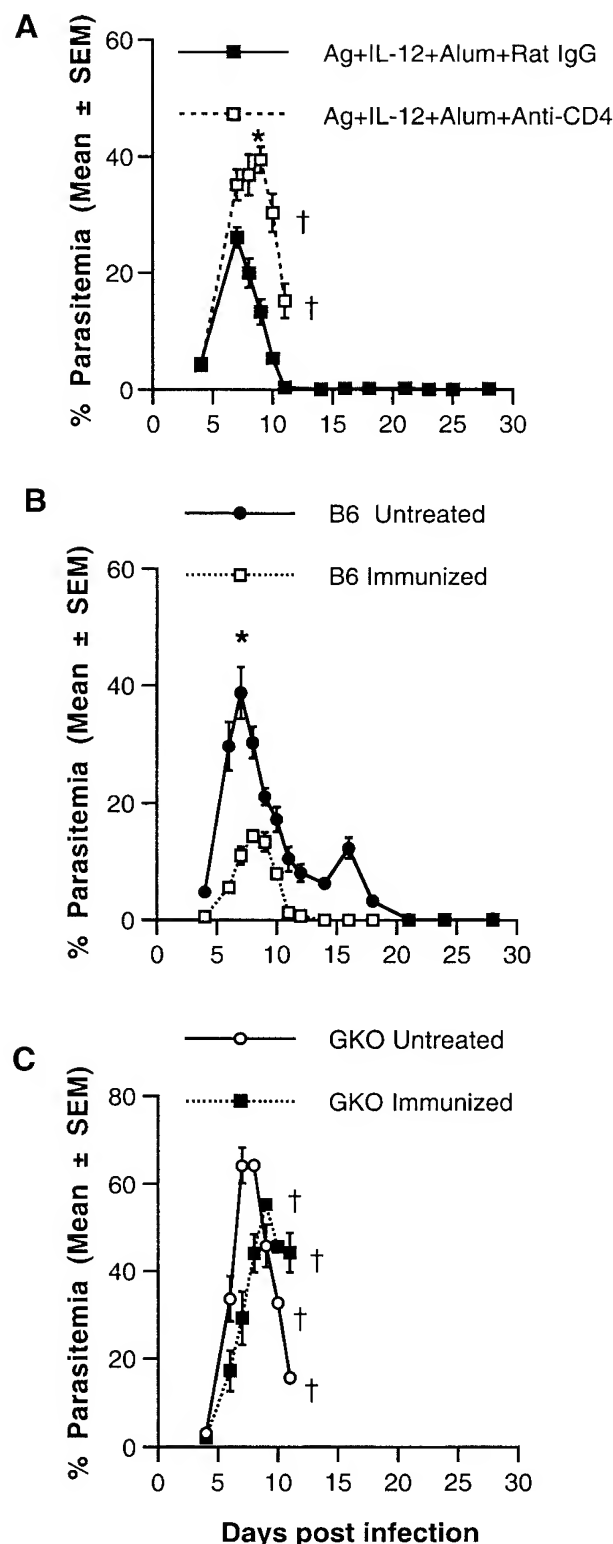


FIG. 3. Course of parasitemia and survival in immunized CD4⁺ T cell depleted A/J mice or in wild-type or GKO C57BL/6 mice. To deplete CD4⁺ in vivo, A/J mice were treated i.p. with GK1.5 monoclonal antibody or with an equivalent amount of rat IgG as a control 3 days prior to challenge infection and three times per week during infection. Two weeks after boosting, mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC and the course of parasitemia was determined (A). Female wild-type (B) and GKO (C) C57BL/6 mice were immunized with antigen (Ag) plus IL-12 in alum, and 2 weeks after boosting,

pared to mice immunized with antigen plus control ODN in alum ($P < 0.001$), and mice in the former group cleared the parasite by 2 weeks postinfection. The combination of antigen plus control ODN in alum was not protective, and 100% of the mice in this group succumbed to challenge infection, with fulminant parasitemia levels by day 10 postinfection.

DISCUSSION

Previous studies demonstrate that coadsorption of antigen and IL-12 to alum promotes both type 1 cytokine and antibody responses (19, 21). Since both cellular and humoral responses have been implicated in protective immunity to malaria, we reasoned that immunization with the combination of malaria antigen and IL-12 coadsorbed to alum may enhance protective immunity to blood-stage malaria. To investigate this possibility, we examined the feasibility of using crude malaria antigen coadsorbed with IL-12 to alum as a vaccine against blood-stage malaria in the mouse model of *P. chabaudi* AS. Cellular and humoral immune responses were compared in A/J mice immunized with antigen plus IL-12 in alum as well as antigen alone, antigen in alum, or antigen plus IL-12 and boosted 3 weeks later with antigen alone prior to challenge infection.

A/J mice are susceptible to primary *P. chabaudi* AS infection and experience fulminant and lethal parasitemia by 10 to 13 days postinfection (36). During the first week of infection, spleen cells from these mice produce high levels of IL-4 and low levels of IFN- γ in vitro in response to the parasite antigen (38). Determination of proliferation and cytokine production in vitro by spleen cells from A/J mice immunized with the various vaccine combinations revealed that spleen cells from mice immunized with malaria antigen plus IL-12 in alum had the highest levels of proliferation as well as of IFN- γ production in response to the specific antigen. Spleen cells from these mice also produced lower levels of the Th2 cytokine IL-4 and the Th1 cytokine TNF- α and low levels of IL-10.

The finding of high levels of production of IFN- γ is consistent with observations in previous studies with IL-12 as an adjuvant together with antigen for vaccination against *L. major* (1), *S. mansoni* (46–48), or *Listeria* (27), which demonstrate that inclusion of this cytokine in the vaccine formulation induces a strongly polarized type 1 cytokine response with production of high levels of IFN- γ . In these studies, the combination of IL-12 and antigen was administered as a vaccine in the absence of alum or another adjuvant. However, Jankovic et al. (19) observed that administration of IL-12 and human immunodeficiency virus type 1 gp120 induces a shift from a type 2 to a type 1 cytokine profile only when coadsorbed to alum. In the case of vaccination against *L. major* (1) or *S. mansoni* (46–48) with the combination of parasite antigen and IL-12, Th2 cytokine production, including IL-4 production, is markedly diminished. Taken together, these findings in various infection mod-

mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC and the course of parasitemia was determined. Similar results were obtained in a replicate experiment using male wild-type and GKO mice. In panel A, the asterisk designates a P value of <0.001 for control versus CD4⁺-T-cell-depleted mice. In panel B, the asterisk designates a P value of <0.0001 for untreated versus immunized C57BL/6 mice.

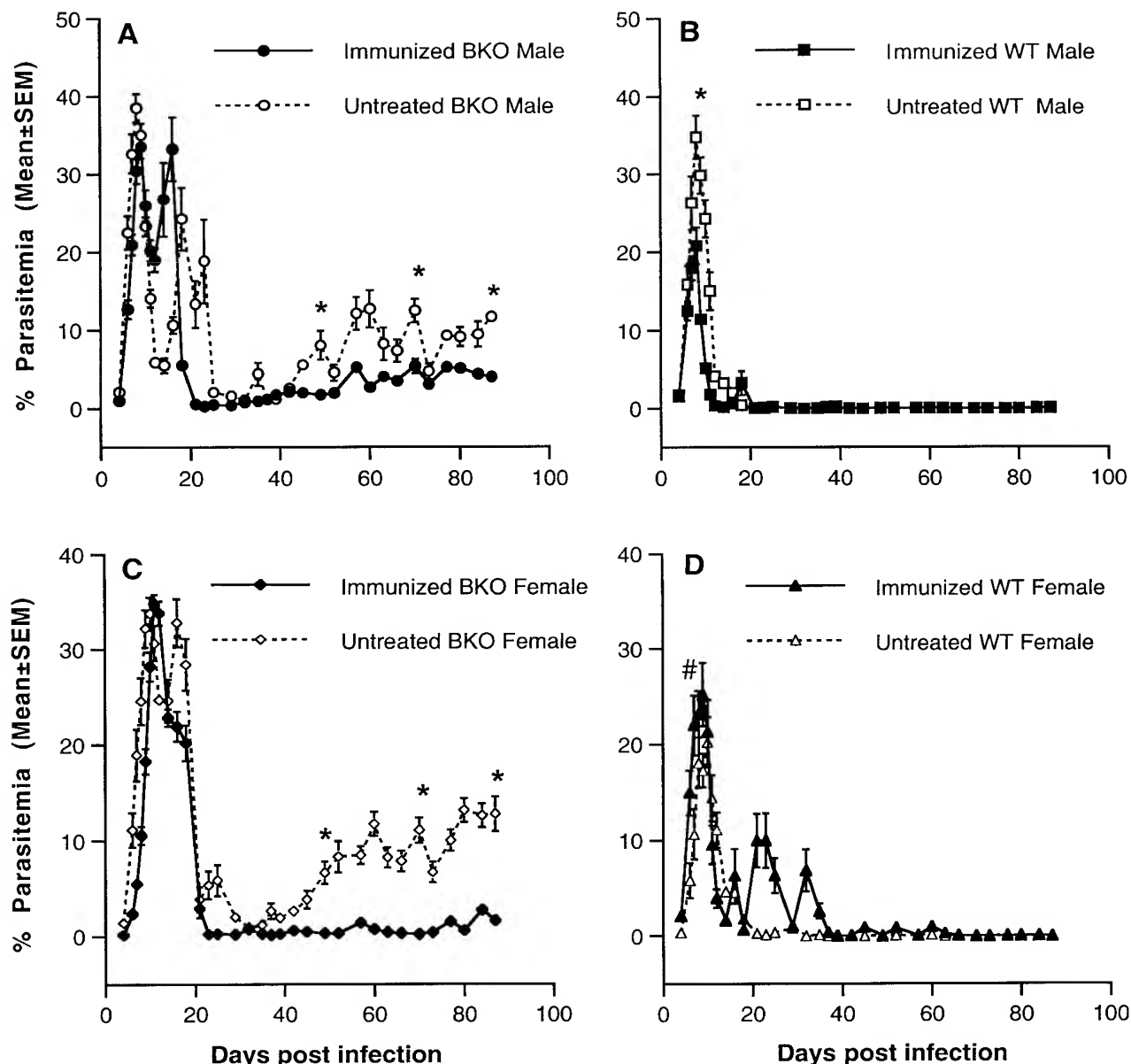


FIG. 4. Course of parasitemia in immunized B-cell-deficient μ -MT (BKO) and wild-type (WT) C57BL/10 mice. Groups of BKO (male, $n = 6$; female, $n = 8$) and WT (male and female, $n = 10$) mice were immunized s.c. with antigen plus IL-12 in alum and boosted i.p. with antigen 3 weeks later. Two weeks later, mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC and the course of parasitemia was determined in male (A and B) and female (C and D) BKO (A and C) and wild-type (B and D) mice. *, $P < 0.001$; #, $P < 0.05$ for nonimmunized versus immunized mice.

els with mice are in accordance with the ability of IL-12 to promote the differentiation of CD4⁺ Th1 cells (12).

Our observations that the combination of malaria antigen plus IL-12 in alum induced high levels of total malaria-specific antibody and IgG2a antibody as well as moderate levels of malaria-specific IgG1 are consistent with previous studies utilizing IL-12 in a vaccine formulation. The present study did not address whether the increased protection seen in the group of mice immunized with the combination of malaria antigen plus IL-12 in alum is due to a particular subclass or isotype of antibody or due to a generalized increase in antibody titers. Wynn et al. (48) demonstrated that mice vaccinated with a combination of IL-12 and irradiated *S. mansoni* cercariae have

significant increases in parasite-specific IgG2a, IgG2b, and IgG1. Studies by Jankovic et al. (19) demonstrated that mice vaccinated with IL-12 and recombinant gp120 envelope protein from human immunodeficiency virus type 1 coadsorbed to alum have high levels of specific IgG2a, IgG2b, and IgG3 isotypes as well as significantly increased gp120-specific IgG1 isotype levels compared to mice immunized with antigen in alum. Interestingly, in these two studies, enhanced IgG1 production occurred in the face of suppressed IL-4 production when IL-12 was included in the vaccine formulation. Similarly, our results indicate that vaccination with the combination of malaria antigen plus IL-12 coadsorbed to alum induced a Th1 immune response in vaccinated mice. The induction of a Th1

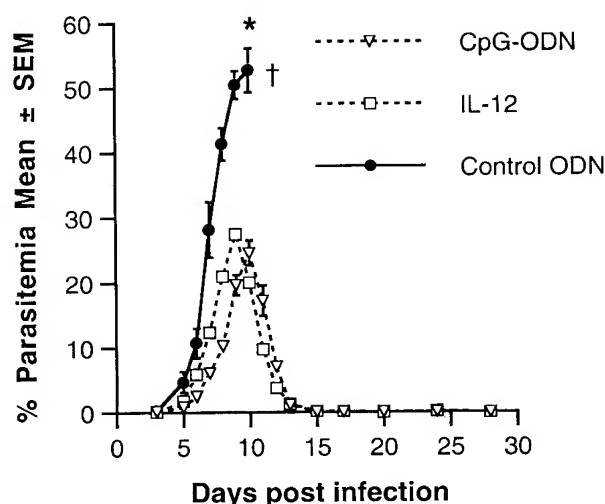


FIG. 5. Course of parasitemia in A/J mice immunized with antigen plus IL-12 in alum or antigen plus CpG-ODN in alum. Groups of 5 A/J mice were immunized s.c. with either antigen plus IL-12 in alum (IL-12), antigen plus CpG-ODN in alum (CpG-ODN), or antigen plus ODN in alum (Control ODN) and boosted i.p. with antigen 3 weeks later. Two weeks later, mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC and the course of parasitemia was determined. *, $P < 0.001$ for day 9 parasitemia between antigen plus ODN in alum versus antigen plus CpG-ODN in alum and $P = 0.114$ for antigen plus CpG-ODN in alum versus antigen plus IL-12 in alum.

immune response by administration of malaria antigen plus IL-12 coadsorbed to alum is relevant given the important role of type 1 cell-mediated and humoral immune responses in mediating naturally induced immunity against malaria in mice infected with blood-stage *P. chabaudi* AS and possibly in humans (24, 29, 39, 40).

Importantly, immunization with the combination of malaria antigen plus IL-12 in alum induced strong protective immunity against challenge infection with blood-stage *P. chabaudi* AS in both susceptible A/J and resistant C57BL/6 mice. In contrast to control A/J mice, which experience a severe course of parasitemia and 100% mortality (36), immunization with either antigen plus IL-12 or antigen plus IL-12 coadsorbed to alum resulted in less severe courses of infection and significant decreases in peak parasitemia levels. However, only mice immunized with antigen plus IL-12 in alum experienced 100% survival. Moreover, the protection induced by this formulation was long-lasting since mice challenged 3 months after boosting were still completely protected against *P. chabaudi* AS. This group of animals had significant decreases in peak parasitemia levels and time to parasite clearance comparable to mice challenged 2 weeks after boosting. In both instances, there was 100% survival of vaccinated mice.

Although $CD4^+$ T cells are known to play an important role in immunity to primary blood-stage *P. chabaudi* AS (24, 29), little is known about the role of these cells in vaccine-induced immunity to blood-stage malaria. Earlier studies by Langhorne and colleagues (25) demonstrated that depletion of $CD4^+$ T cells from immune C57BL/6 mice results in a low, transient parasitemia following challenge with *P. chabaudi* AS, which is eventually cleared. In contrast, our results in $CD4^+$ -T-cell-depleted, immunized mice indicate that $CD4^+$ T cells play a

critical role in immunity induced by vaccination with malaria antigen and IL-12 in alum. We observed that immunized $CD4^+$ -T-cell-depleted mice experienced severe and lethal infections when challenged with *P. chabaudi* AS.

It is likely that $CD4^+$ T cells participate in immunity induced by immunization with malaria antigen and IL-12 coadsorbed to alum by producing IFN- γ , although the studies performed here did not address the cellular source of this cytokine in immunized mice. Both $CD4^+$ T cells and NK cells were found to produce IFN- γ in mice vaccinated with *L. major* antigen and IL-12 (1). NK cells may also be a source of IFN- γ in mice immunized with malaria antigen and IL-12 in alum. NK cells have been found to produce IFN- γ early in infection with various species of mouse malaria parasites, including *P. chabaudi* AS (8, 28). Recent studies with humans demonstrated that *Plasmodium falciparum*-infected red blood cells induce IFN- γ production by NK cells from individuals infected with *P. falciparum* and nonexposed donors (3). IFN- γ is considered to be a major component of innate and acquired immunity to primary blood-stage *P. chabaudi* infections (11, 24, 40, 42). The inability to protect GKO mice, compared to wild-type C57BL/6 mice, against challenge infection as shown here indicates that IFN- γ is also a critical cytokine in vaccine-induced immunity following immunization with malaria antigen and IL-12 coadsorbed to alum. In humans, IFN- γ production has been found to correlate with resistance to reinfection with *P. falciparum* as well as with protection from clinical attacks of malaria (6, 9, 26). Based on these observations, it has been concluded that IFN- γ production should be considered an important hallmark of effector T-cell function for the development of an effective malaria vaccine (14, 32). Our results in the present report support this contention.

During primary *P. chabaudi* AS infection, mice rendered B-cell deficient by treatment from birth with anti-IgM antibodies or μ -MT mice with targeted disruption of the membrane exon of the immunoglobulin μ -chain gene can control acute parasitemias in a manner similar to that of intact mice (41, 44). However, B-cell-deficient mice maintain a chronic low level of parasitemia, indicating that effective parasite clearance at the later, chronic stage of infection requires the presence of B cells (41, 44). In addition to their ability to produce antibody, B cells may also play a role via production of IL-10 (41) in the switch from Th1 cells producing IFN- γ , which mediates control of acute parasitemia, to Th2 cells which provide help for antibody production leading to clearance of primary blood-stage *P. chabaudi* AS infection. Studies in μ -MT mice also showed that B-cell-deficient animals are unable to control a challenge infection and develop parasitemia levels similar in magnitude to a primary infection (44). These findings suggest that B-cell-dependent mechanisms may be important for an effective memory response to *P. chabaudi* AS infection (44). In the present study, we observed that immunization of B-cell-deficient μ -MT mice with malaria antigen and IL-12 coadsorbed to alum is ineffective in providing enhanced protection against challenge infection with *P. chabaudi* AS, suggesting a role for a B-cell-dependent mechanism(s) in vaccine-induced immunity.

We also examined the possibility of replacing IL-12 with immunostimulatory CpG-ODN. Because of its ability to induce a type 1 pattern of cytokine production dominated by IL-12

and IFN- γ with little secretion of type 2 cytokines, CpG-ODN have been found to be useful as adjuvants for vaccines, including peptide vaccines, against a variety of pathogens (4, 5, 7, 15, 23, 30, 35, 45). Near and colleagues (30) recently demonstrated that vaccination with the combination of CpG-ODN and *P. yoelii* MSP1₁₉ in alum resulted in a dramatic elevation of IFN- γ production as well as elevated production of IL-10 by MSP1₁₉-stimulated splenocytes, suggesting induction of a mixed Th1 and Th2 response. In mice vaccinated with this formulation, IgG1 was found to be the predominant antibody isotype in sera, although increased levels of MSP1₁₉-specific IgG2a, IgG2b, and IgG3 isotype antibodies were also observed. Furthermore, increased antibody levels were found to correlate with protection against challenge infection with a high dose of *P. yoelii* PRBC. Our experimental results demonstrate that inclusion of immunostimulatory CpG-ODN instead of IL-12 in the vaccine formulation provided strong protection against blood-stage *P. chabaudi* AS infection in A/J mice. Studies in progress in our laboratory demonstrate that immunization with CpG-ODN and crude malaria antigen in alum induces high levels of malaria-specific IgG2a in A/J mice before challenge infection in comparison to immunization with control ODN and antigen in alum (unpublished data).

In conclusion, the results of this study illustrate that it is possible to enhance the potency of a crude malaria antigen in alum vaccine formulation by inclusion of agents with immunostimulatory properties, such as IL-12 or CpG-ODN. Although alum is the most commonly used adjuvant and is approved for use by humans by the U.S. Food and Drug Administration, our data indicate that this adjuvant is weak in promoting vaccine-induced protective immunity against blood-stage malaria. Furthermore, immunity induced by immunization with malaria antigen and IL-12 coadsorbed to alum induced a long-lasting, Th1 immune response required for protection against challenge infection with *P. chabaudi* AS infection.

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Mitogenic synthetic polynucleotides suppress the antibody response to a bacterial polysaccharide

D.S. Threadgill*§, L.L. McCormick†, T.L. McCool†, N.S. Greenspan† and J.R. Schreiber*†‡

Unmethylated bacterial DNA containing a high frequency of the CpG motif, is mitogenic and induces T-cell independent, murine B-cell proliferation. These stimulatory effects are also induced by synthetic oligonucleotides that contain one or more unmethylated CpG dinucleotides (CpG oligo). Such mitogenicity is not seen with highly methylated vertebrate DNA, which has a lower prevalence of the CpG motif than bacterial DNA. Due to their stimulatory effects, CpG oligo have been proposed for use as vaccine adjuvants. In order to determine if a synthetic CpG oligo that was stimulatory for B-cell proliferation could augment the murine antibody response to protective bacterial polysaccharide epitopes (Pseudomonas aeruginosa LPS-O polysaccharide side chain; high-molecular-weight polysaccharide or high-MW PS), BALB/c mice were injected with mitogenic doses of CpG oligo simultaneously with high-MW PS, and antibody titers were measured by ELISA weekly for 4 weeks. Controls received PBS, a nonstimulatory control oligo plus PS, CpG alone, or PS alone. Despite evidence of B-cell mitogenicity and an increase in total IgM in CpG oligo-treated mice, CpG oligo treatment plus PS significantly decreased the high-MW PS antibody response compared to PS alone. The blunting of the anti-PS antibody response could be eliminated by vaccinating the animals with PS prior to CpG oligo. We conclude that despite in vitro and in vivo evidence of B-cell proliferation, this CpG oligo reduces PS-specific antibody responses in an animal model when given simultaneously with a bacterial polysaccharide. Based on results in this model, oligonucleotides containing stimulatory unmethylated CpG dinucleotides may not be useful adjuvants when given simultaneously with bacterial PS vaccines. © 1997 Elsevier Science Ltd.

Pseudomonas aeruginosa continues to be a significant human pathogen despite the availability of appropriate antimicrobial agents. Specific groups of individuals such as neutropenic cancer patients, individuals with burns, and children with cystic fibrosis are particularly susceptible to serious infections with *Pseudomonas aeruginosa*^{1,2}. Antibodies directed to certain surface polysaccharides (PS) of *Pseudomonas*, in conjunction with phagocytes, are often protective against infection with this organism. Antibodies against lipopoly-

saccharide O-specific side chain (high-MW PS) for example, supply serotype-specific protection³.

The immune response to bacterial polysaccharides differs from immune responses to protein antigens. Polysaccharide antigens are 'T-independent' in character, do not commonly elicit booster responses with repeat immunization, and elicit antibodies of restricted clonotype and isotype (IgM and IgG3 in mice, IgM, IgG2 and IgG1 in humans)^{4,5}. In addition, polysaccharide antigens are particularly poor immunogens in small children under the age of two years and in immunocompromised adults. Conjugation of bacterial polysaccharides to proteins results in immunogens that act as partially 'T-dependent' antigens, and have greatly improved immunogenicity for some bacterial polysaccharides such as the capsule of *Haemophilus influenzae* type b^{6,7}. Current polysaccharide-protein conjugate vaccines, however, require multiple doses to achieve protective antibody levels in children. In addition, some polysaccharides from bacterial pathogens are poor immunogens even when conjugated to appropriate carrier proteins^{8,9}.

*Department of Pediatrics, Case Western Reserve University School of Medicine, Rainbow Babies and Children's Hospital, Cleveland, OH 44016, USA.†Department of Pathology, Case Western Reserve University School of Medicine, Rainbow Babies and Children's Hospital, Cleveland, OH 44106, USA.§Present address: Department of Medicine, Division of Infectious Diseases, Vanderbilt University, Nashville, Tennessee. ‡Corresponding author. Phone (216) 844-3645, Fax (216) 844-8362. (Received 11 April 1997; revised version received 28 May 1997; accepted 28 May 1997)

Thus adjuvants that could enhance the anti-polysaccharide antibody response could have important clinical benefit.

Bacterial DNA has recently been proposed as a possible adjuvant for polysaccharide antigens and other poorly immunogenic compounds. Bacterial DNA has been shown to be a potent activator for murine splenocytes, including B cells, NK cells, and CD4⁺ T cells in both in vitro and in vivo experiments¹⁰⁻²⁰. By contrast, vertebrate DNA treatment is not stimulatory in these models. Oligonucleotides containing nonmethylated CpG motifs not normally present in vertebrate DNA but frequently occurring in bacterial DNA, have also been shown to activate lymphoid cells^{10-13,15,17,21-23}. In particular, when purified splenic B cells were cultured with CpG, nonCpG or nonstimulatory CpG oligos (NSCpG), tritiated thymidine and tritiated uridine incorporation was dramatically increased only in CpG-treated B-cells^{13,15,17}. This evidence of in vitro activation was further demonstrated by data showing an increase in IgM secretion by CpG-treated B lymphocytes^{13,20}. Finally, short-term in vivo experiments involving intraperitoneal injection of mice with phosphorothioate CpG, nonCpG or NSCpG oligos followed 24 h later by splenectomy, demonstrated that CpG-treated B cells had been induced in vivo to proliferate¹³. As a result of these in vitro and other short-term in vivo results, some investigators have suggested that oligonucleotides containing nonmethylated stimulatory CpG motifs could be used as adjuvants for inducing an improved immune response to normally poor immunogens¹³. The current study was performed to determine if the simultaneous administration of stimulatory CpG oligo and high-MW PS could increase the anti-PS antibody response in an animal model.

MATERIALS AND METHODS

Bacterial polysaccharide

The high molecular weight polysaccharide (high-MW PS) component of Fisher-Devlin immunotype 1 *Pseudomonas aeruginosa* lipopolysaccharide O-specific side chain was isolated from bacterial culture supernatants as previously described²⁴. This material contains the immunodeterminants of the O-specific side chain but does not contain lipid A or nucleic acids; it was kindly provided by Dr. Gerald Pier, Channing Laboratories, Harvard Medical School, Boston.

Phosphorothioate DNA oligonucleotides

Unmethylated oligonucleotides including either a stimulatory CpG motif (CpG) or control nonstimulatory CpG motif (NSCpG) were purchased from Operon (Alameda, CA). The two oligonucleotide sequences were chosen on the basis of previous reports of their immunostimulatory properties and were:

- (1) stimulatory CpG oligo '1d' 5' GCATGACGTT-GAGCT 3' so that the CpG motif is flanked by two 5' purines and two 3' pyrimidines;
- (2) nonstimulatory CpG oligo '1a': 5' GCTAGATGT-TAGCGT 3' containing a CpG motif but flanked by two 5' purines and only one 3' pyrimidine.

These sequences have been previously demonstrated to be stimulatory or nonstimulatory, respectively, to mammalian B cells^{13,20}.

Animals

8-14-week-old female BALB/c ByJ pathogen-free mice (Jackson Laboratories, Bar Harbor, Maine) were maintained in a *Pseudomonas*-free animal facility. Drinking water was filtered and autoclaved, and bedding, food and microisolator cages were autoclaved before use. We have previously shown that animals remaining in this facility do not become pseudomonas-colonized and do not seroconvert^{3,25}.

Long-term animal experiments (duration 1 month)

The BALB/cByJ mice were bled via tail-vein puncture and then injected intraperitoneally with either phosphate-buffered saline (PBS), 10 µg high-MW PS, 500 µg NSCpG or CpG oligo or a combination of oligonucleotide plus polysaccharide (either 250 µg or 500 µg oligo plus 10 µg high-MW PS). Mice were bled once weekly for 4 weeks, and sera were analyzed for anti-high-MW PS antibodies by ELISA, as described below (Section 1.5)³.

Enzyme-linked immunosorbent assay (ELISA) to detect anti-high-MW PS antibodies

ELISA was used to detect anti-high-MW PS antibodies in mouse sera as we have previously described^{3,25}. High-MW PS was tyraminated via cyanogen bromide coupling of tyramine to the PS in order to enhance binding to the ELISA plate^{3,24,25}. Briefly, tyraminated PS was coated on 96-well microtiter plates (Immunlon 4, Dynatech) at a concentration of 1 µg ml⁻¹ (100 µl well⁻¹) overnight at 4°C. Plates were washed with PBS-Tween and blocked with 1% BSA in PBS. After three additional PBS-Tween washes, mouse sera (diluted 1:100) were loaded (100 µl well⁻¹) and incubated overnight at 4°C. Goat anti-mouse Ig (H+L) alkaline phosphatase conjugate (AP; Fisher/Southern Biotech, Atlanta, GA) was diluted 1:1000 and added to washed plates. After excess conjugate was washed off, plates were developed with diethanolamine (DEA) buffer and *p*-nitrophenol phosphate (pNPP, Sigma, St. Louis, MO) using standard procedures, and then read on an ELISA plate reader (Dynatech) at 410 nm. Normalization of data from each plate was performed using a standard curve based on binding of a murine monoclonal antibody specific for high-MW PS that we have previously described³. The average slope of all the monoclonal antibody standard curves for an experiment was calculated and then used to normalize the data, thus minimizing plate-to-plate variation. Once raw data were normalized, the mean OD per treatment group for each time point was calculated. Standard error of the mean (SEM) was used to generate error bars for graphical comparisons of the mean ODs. The seroconversion frequency defined as the number of animals having OD measurements of threefold or greater than the background of the PS-specific ELISA divided by the total number of animals in that treatment group, was also determined. χ^2 analysis was utilized to compare the seroconversion frequencies of the oligo treatment groups to the PS alone treatment group.

ELISA to measure total serum IgM

ELISA was used to measure total IgM levels in mouse sera. Briefly, goat anti-mouse Ig (H+L; Fisher/Southern Biotech) was coated on microtiter plates (Corning) at a concentration of $5 \mu\text{g ml}^{-1}$ ($100 \mu\text{l well}^{-1}$) overnight at 4°C . Washed plates were blocked with 1% BSA, washed three times and loaded with mouse serum samples (dilution 1:10000, $100 \mu\text{l well}^{-1}$) for overnight incubation at 4°C . After three washes, goat anti-mouse IgM AP (Fisher/Southern Biotech) diluted 1:1000 was added to the plates. Plates were developed with DEA buffer and pNPP using standard procedures, and read on a Dynatech ELISA plate reader at 410 nm. Conversion of optical density (OD) measurements to mg ml^{-1} IgM was based on IgM standard curves generated for each ELISA plate with a purified murine IgM standard (Fisher/Southern Biotech). Mean IgM levels \pm standard error of the mean (SEM) were calculated for each treatment group at each time point.

Short-term animal experiments (duration 1 day)

BALB/c mice were injected intraperitoneally with the same reagents used for the long-term experiments, but were sacrificed after 24 h. Splenocytes were isolated from spleens and stained for flow-cytometric analysis as described below (Section 1.8).

Two-color flow cytometric analysis of splenocytes from immunized mice

Spleens from mice receiving various immunizations were pressed between frosted microscope slides to release the splenocytes. Debris was allowed to settle and then the supernatant was collected and centrifuged. Red cells were lysed prior to antibody staining according to standard procedures^{26,27}. Antibodies selected for staining of splenocytes included fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgM (Fisher/Southern Biotech), FITC-labeled mouse anti-mouse Ia_d (PharMingen, San Diego, CA), FITC-labeled hamster anti-mouse CD3 ϵ (PharMingen) and phycoerythrin (PE)-labeled rat anti-mouse CD45R (B220; PharMingen). Control staining was also performed with FITC- or PE-labeled isotype-matched antibodies. Additionally, rat anti-mouse CD32/CD16 (Fc blockTM, PharMingen) was used prior to incubation of splenocytes with the staining reagents to decrease background staining. Antibodies were used so that $50 \mu\text{l}$ of the Fc blocking antibody at $20 \mu\text{g ml}^{-1}$ was added to $50 \mu\text{l}$ of splenocytes at $2 \times 10^7 \text{ cells ml}^{-1}$ for 5 min on ice, followed by addition of the same concentration of staining antibodies for 30 min on ice. Cells were double-stained with anti-B220 and either anti-IgM, anti-Ia^d or anti-CD3 ϵ as appropriate. After two washes, cells were analyzed on a Becton Dickinson FACScan flow cytometer.

RESULTS

Detection of antibodies to LPS O-side chain polysaccharide in CpG oligo and polysaccharide-immunized mice

The adjuvant activity of CpG oligonucleotide was assessed by immunizing mice with a bacterial

polysaccharide (high-MW PS) in combination with CpG as well as NSCpG oligonucleotides. Antigen-specific ELISA was then performed on sera from immunized mice. Simultaneous immunization of mice with CpG oligo and *Pseudomonas aeruginosa* LPS O-side chain polysaccharide (high-MW PS) yielded lower antibody titers than control animals given nonCpG oligo plus polysaccharide or polysaccharide alone (Figure 1A and B). NSCpG oligo plus PS-immunized mice also exhibited slightly decreased antibody titers (Figure 1A and B), however, these titers were substantially higher than the CpG oligo plus PS-immunized mice. In addition to decreased antibody titers, CpG oligo plus PS-immunized mice seroconverted (defined as an increase in ELISA OD to three times the background) to polysaccharide significantly less frequently than animals immunized with PS and NSCpG oligo or with PS alone (Table 1). Thus, use of CpG oligo simultaneously with an immunogenic bacterial PS resulted in a decreased titer of PS-specific antibody and in a reduction in frequency of seroconversion. These results were unexpected because of the mitogenic activity of CpG oligonucleotides for mouse B cells.

Measurement of serum IgM in immunized mice

Total serum IgM levels were measured to determine if CpG oligo was appropriately stimulatory to B cells in

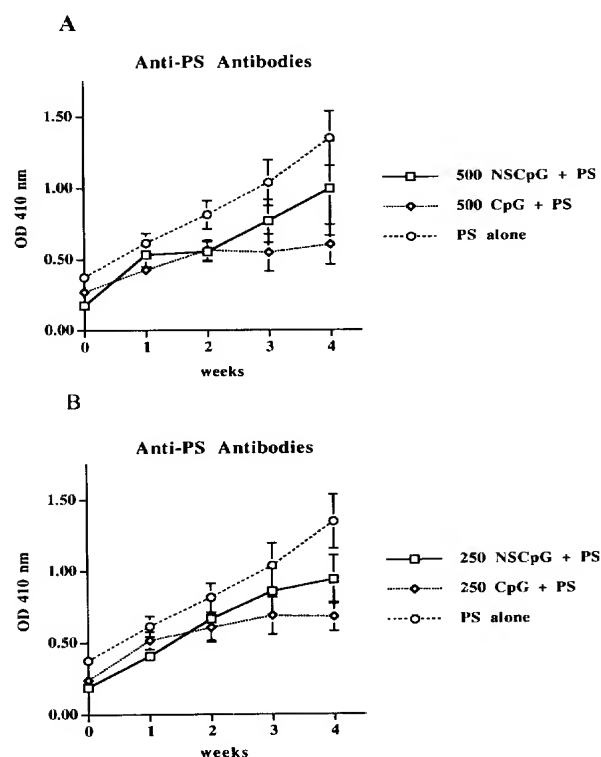


Figure 1 The effect of CpG oligo administration on seroconversion of mice to *Pseudomonas aeruginosa* high-MW PS. An antigen-specific ELISA described in Section 1.5 was used to evaluate sera for antibodies to PS. Mice were immunized with $10 \mu\text{g}$ PS either alone or in combination with $500 \mu\text{g}$ NSCpG or CpG oligo (A) or in combination with $250 \mu\text{g}$ NSCpG or CpG oligo (B). Each treatment group contained five mice, and points represents means from three different experiments

Table 1 CpG oligo administration decreases the percentage of animals seroconverting to *Pseudomonas aeruginosa* high-molecular-weight polysaccharide

Treatment	Number of mice (% seroconversion)
PBS	0/10 (0)
PS	15/15 (100)
500CpG+PS	7/15 (53) ^a
250CpG+PS	3/10 (30) ^b
500NSCpG+PS	13/16 (81)
250NSCpG+PS	9/10 (90)
PS/PBS ^c	3/3 (100)
PS/250CpG ^c	3/3 (100)
PS/250NSCpG ^c	3/3 (100)

%Seroconversion = (# of animals whose OD at 410 nm reached 3 × the background for the ELISA/# of animals receiving the treatment) × 100. Data were compared by χ^2 analysis to determine significance.

^a $P < 0.0025$ versus PS.

^b $P < 0.0001$ versus PS.

^cAnimals were immunized with polysaccharide followed 48 h later by PBS, CpG or non-CpG oligos.

vivo in our experimental system as previously described¹³. Total IgM levels were increased in CpG oligo-immunized animals as compared to animals given PBS or NSCpG oligo, consistent with B-cell mitogenicity of the CpG oligo in vivo (Figure 2A). In addition, animals simultaneously treated with 500 μ g CpG oligo

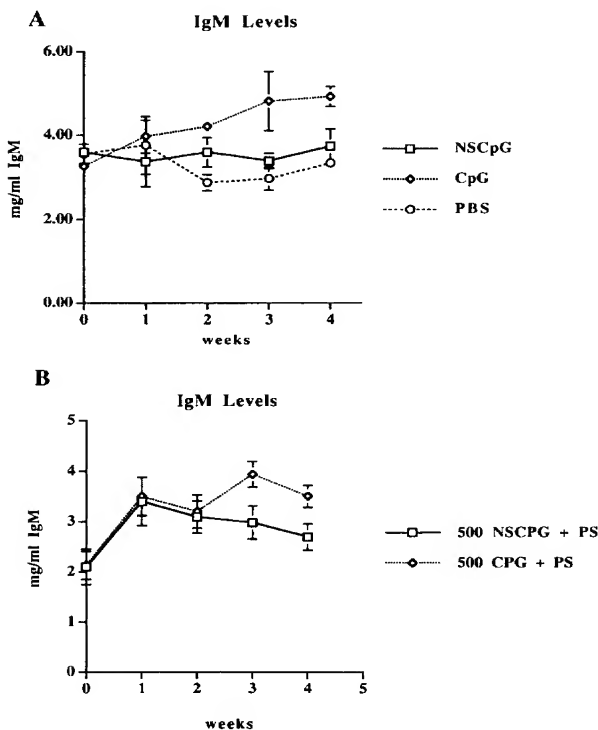


Figure 2 (A) The effect of CpG oligo administration on the total serum IgM levels in mice. Mice were immunized with PBS, NSCpG oligo or CpG oligo (500- μ g dose only). An isotype-specific ELISA was used to evaluate mouse sera for total IgM (Section 1.6). The IgM levels were higher in mice immunized with 500 μ g CpG oligo compared to mice immunized with NSCpG or PS alone. (B) Total serum IgM of mice immunized with CpG oligo simultaneously with PS was also increased compared to NSCpG plus PS controls. The data represent means from three experiments with a total of 15 mice per treatment group

plus PS had elevated total IgM levels compared to animals immunized simultaneously with 500 μ g NSCpG oligo plus PS (Figure 2B). Thus, the increased total IgM levels in mice treated simultaneously with CpG oligo and bacterial PS suggests that failure to upregulate B cells or interference of PS with CpG oligo mitogenicity was not the explanation for the depressed PS-specific antibody levels.

Flow cytometry analysis of BALB/c mouse splenocytes after in vivo exposure to either nonCpG or CpG oligo

Because experiments over four weeks showed a suppressive effect of CpG oligo on polysaccharide-specific antibody levels, flow cytometry experiments were performed in order to determine whether the CpG oligo was acting appropriately as a B-cell mitogen in our experimental systems. Short-term animal experiments (i.e. splenocytes collected 24 h after vaccination with oligo or PBS) confirmed that the CpG oligo was a B-cell mitogen in our experiments. The number of B cells in CpG-treated mice was increased as assessed by IgM and Ia^d expression (Figure 3, Table 2). Additionally, the mean fluorescence measurement of Ia^d was much higher in CpG-treated mice, indicating an increase in the surface expression of this marker (Table 2). Splenocytes from animals simultaneously treated with PS and CpG oligo showed a similar pattern to that seen with CpG oligo treatment alone, indicating that the PS was not affecting the ability of the CpG oligo to perform as a B-cell mitogen in vivo (Table 2).

Polysaccharide pretreatment to assess the importance of timing of oligonucleotide vaccination to the anti-polysaccharide antibody response

To investigate whether the antigen-specific suppression seen in four-week-long animal experiments was the consequence of the simultaneous administration of the polysaccharide antigen with the polyclonal stimulator (i.e. CpG oligo), animals were first immunized with PS, and then vaccinated with CpG oligo 48 h later. Animals immunized first with PS followed 48 h later by CpG oligo were more likely to seroconvert to PS than those simultaneously immunized with CpG oligo and PS (Figure 4, Table 1). In addition, more animals in the groups receiving PS first followed by CpG oligo seroconverted to PS (100%) compared to mice simultaneously immunized with PS and 500 μ g CpG or 250 μ g CpG (53% and 30% seroconversion respectively). Finally, over the four-week experiment, the mean high-MW PS antibody titers in the animals first immunized with PS followed later by CpG oligo was higher than those seen in mice receiving PS and CpG simultaneously (Figure 1A and B and Figure 4).

DISCUSSION

Synthetic oligonucleotides that are CpG-rich and unmethylated, thus mimicking bacterial DNA, have been suggested as adjuvants and biological response modifiers due to their immunostimulatory properties¹³. Previous studies with bacterial DNA or CpG oligos focused primarily on their ability to stimulate in vitro cultures of murine splenocytes and human lymphocytes¹⁰⁻²³. More recently, some investigators have shown

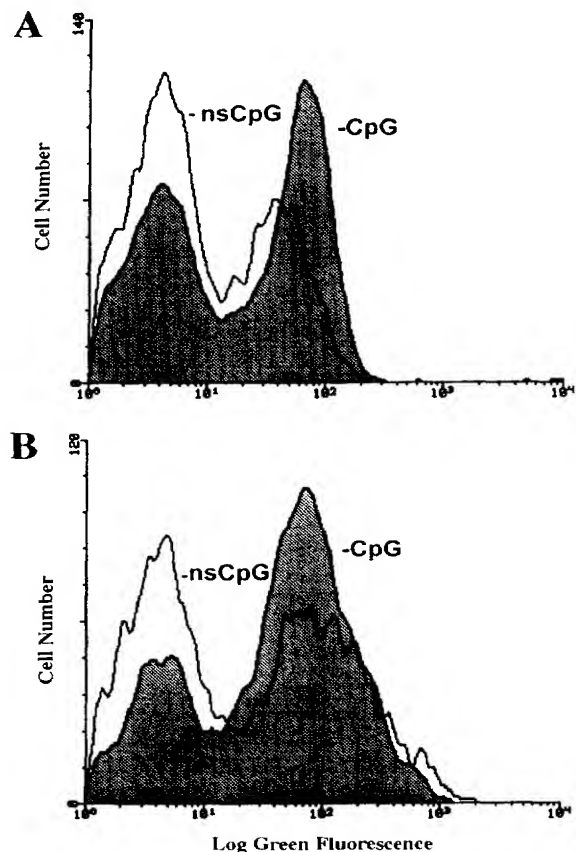


Figure 3 Flow cytometry analysis demonstrating the mitogenic effect of CpG immunization on mouse splenic B cells. Total B cell number was increased in the CpG oligo-immunized mice but not in control mice given NSCpG oligos. Panel A shows the staining of mouse splenocytes from immunized animals by FITC-labelled mouse anti-mouse class II (Ia^d). Panel B shows the staining of mouse splenocytes by FITC labelled goat anti-mouse IgM. For both panels, shaded histograms depict staining for CpG oligo-immunized mice, and open histograms staining for NSCpG oligo recipients. The experiment was repeated three times with similar results. PS addition to the oligo injection mixture did not affect the mitogenic properties of the oligos, and PS alone was not mitogenic in the assay (not shown)

Table 2 Effects of CpG oligo and PS immunization on proliferation and class II MHC expression of mouse splenic B cells

Treatment	B cells/T cells	Mean channel fluorescence Ia ^d
PBS	0.517	36.39
500NSCpG	0.560	38.53
500CpG	0.786	46.37
PS	0.544	32.96
250NSCpG+PS	0.501	32.49
250CpG+PS	0.718	41.37

The B- to T-cell ratios were calculated based on the number of cells staining with α -B220 (B cells) and the number of cells staining with α -Cd3 ϵ (T cells). The ratios were not different for cells stained with α -IgM and α -Ia^d. The results indicated are from one animal per treatment group. With the exception of the PS-alone treatment group, two or more animals were evaluated for each treatment.

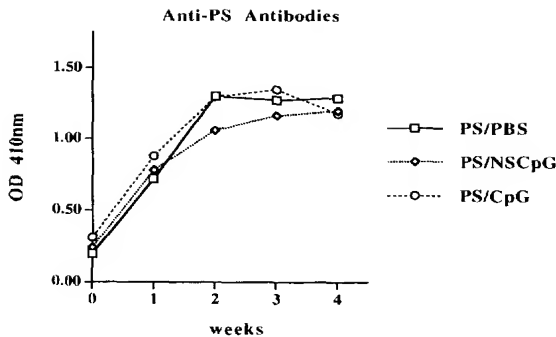


Figure 4 Immunization of mice with high-MW PS prior to CpG oligo restores the PS-specific antibody response. Mice were administered 10 μ g PS followed 48 h later by administration of PBS, NSCpG or CpG oligo (250- μ g dose). The PS-specific ELISA was then used to evaluate sera for antibody titer to PS. Each treatment group contained three mice

synthetic oligos are B-cell mitogens in vivo and yield increased production of a variety of cytokines as well as IgM^{10,13,20}. These experiments only examined short-term (1–3 days) immune responses in mice and did not investigate the effect of CpG oligos on pathogen-specific immunity. In the present study, we focused on a longer term (1 month) in vivo mouse model for the antigen specific seroconversion to the high-MW PS component of Fisher–Devlin immunotype 1 *Pseudomonas aeruginosa* lipopolysaccharide O-side chain³. In this model, adult BALB/c mice seroconvert to high-MW PS after one immunization, and the antibodies persist for more than 1 month. We hypothesized that immunization of mice with CpG oligo simultaneously with PS would elevate the levels of PS-specific antibody produced as compared to mice immunized with PS alone or with the nonstimulatory NCpG oligo plus PS, since CpG oligo was an effective mitogen for B cells. In contrast to this expectation, polysaccharide-specific antibody levels were reduced in the mice coadministered CpG oligo and high-MW PS, as compared to mice administered high-MW PS with NSCpG oligo or PS alone without ‘adjuvant’. Two different doses of CpG oligo were suppressive in terms of the number of animals seroconverting to PS and in the titer of anti-PS antibody produced.

Thus, results from in vivo experiments in which mice were immunized simultaneously with high-MW PS from *Pseudomonas aeruginosa* and CpG oligo yielded unexpected results over the 4-week duration of the experiment. Rather than finding CpG oligo to be an effective adjuvant for a bacterial polysaccharide antigen, we found suppression of PS-specific antibody levels, and a significantly decreased frequency of seroconversion. Since bacterial infection in humans exposes the host to bacterial surface PS and bacterial DNA, it seems possible that the nonspecific mitogenicity of bacterial DNA is not optimal for an immune response to surface polysaccharides and may be a bacterial adaptation to evade host defenses. Alternatively, the polyclonal stimulation of mammalian lymphocytes in response to bacterial DNA or nonmethylated CpG oligos may be unrelated to host–pathogen interactions, but rather may result from differences in evolutionary pathways in which vertebrates, unlike prokaryotes, followed a path to CpG

motif suppression in their DNA. Further experiments will be needed to define fully the mechanism of CpG-mediated LPS O-side chain antibody suppression, and to determine if the reduced titers of anti-PS antibodies are seen when other bacterial PS are used as vaccines with CpG oligos. Interestingly, NSCpG oligo immunization with PS resulted in a slight decrease in serum anti-PS antibody titer as well as a nonsignificant decrease in the frequency of mouse seroconversion, suggesting that this DNA oligo may have some immunomodulatory effect. This oligo was chosen because it was nonstimulatory in previous experiments. However, it does contain a CpG motif that is not flanked by two 3' pyrimidines needed for optimal immunostimulatory effect¹³. It is therefore possible that some immunomodulation may have occurred utilizing this control oligo not detected in our in vitro experiments. Additional immunization experiments in animals utilizing stimulatory CpG, nonstimulatory CpG as well as nonCpG oligos should help provide the information necessary to fully evaluate the potential of stimulatory CpG oligos as adjuvants for immunization with bacterial PS.

Once polysaccharide-specific antibody levels were determined to be suppressed in PS/CpG-immunized mice compared to PS-alone and PS/NSCpG-immunized mice, total IgM levels were evaluated. Total serum IgM levels in CpG-immunized mice (both with and without PS) were elevated in comparison to NSCpG immunized (also with and without PS) mice. These results are consistent with CpG oligos being mitogenic in these in vivo studies and suggest that the reduction in PS-specific antibody titers was not due to global immune suppression.

Short-term animal experiments, which measured the number of splenic B cells 24 h after immunization of mice with CpG and NSCpG oligos confirmed previous results that CpG oligos are B-cell mitogens. The coadministration of PS and CpG oligo in these experiments did not result in changes in mitogenicity of the CpG oligo. Additionally, the PS alone was not mitogenic which is consistent with the previously reported low level of nucleic acid and LPS contamination of the high-MW PS preparations and previous data with purified carbohydrate antigens^{3,5,24}.

The broad polyclonal immunostimulatory effects of bacterial DNA and CpG oligos may not be ideal for simultaneous production of anti-bacterial PS antibodies. CpG oligos have been found to increase production of cytokines such as IL-6, a cytokine response that may not be optimal for antibody responses to T-cell independent type 2 bacterial polysaccharide antigens²⁰. IL-6 is known to be a potent inducer of B-cell differentiation and may unfavorably alter B-cell interactions with polysaccharide antigens. Alternatively, increased surface IgM as a result of B-cell exposure to CpG oligo may increase receptor occupancy by the PS on the surface of the B cell and induce tolerance. Overly restrictive crosslinking of B-cell surface receptors has recently been shown to induce tolerance in hapten-specific B cells²⁷. It is also possible that timing of the use of CpG oligo may be important so that use of oligo after PS immunization might stimulate B cells already committed to PS-specific antibody production and thus be an

effective adjuvant. Our data showing recovery of the PS-specific antibody titer if CpG oligo are utilized after immunization with PS support this premise.

Thus, further experiments will be needed to define fully the mechanism of CpG-mediated LPS O-side chain antibody suppression, to determine whether suppression is seen when other bacterial PS or nonPS antigens are used with the CpG oligos, and to determine if different timing of the use of CpG oligo immunization can enhance the anti-PS antibody response. It will also be important to determine if IL-6 or other CpG-induced mediators cause an increase or decrease in B-cell responsiveness to PS antigens. Additional immunization experiments in animals should provide the information necessary to evaluate more fully the potential of CpG oligos as vaccine adjuvants.

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CpG oligonucleotides improve the protective immune response induced by the anthrax vaccination of rhesus macaques

Dennis M. Klinman^{a,*}, Hang Xie^a, Stephen F. Little^b,
Debra Currie^a, Bruce E. Ivins^b

^a Section of Retroviral Immunology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bldg. 29A, Rm. 3 D 10, Bethesda, MD 20892, USA

^b Bacteriology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA

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Abstract

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjuvants, improving the immune response elicited by co-administered vaccines. Combining CpG ODN with anthrax vaccine adsorbed (AVA, the licensed human vaccine) increased the speed, magnitude and avidity of the resultant anti-anthrax response. The protective activity of these Abs was established by passive transfer to anthrax-challenged mice. The ability of CpG ODN to accelerate and magnify the immune response to AVA suggests this strategy may contribute to the development of prophylactic and therapeutic vaccines against biothreat pathogens.

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Keywords: CpG oligonucleotide; Anthrax; Vaccine adjuvant

1. Introduction

Anthrax is caused by the gram-positive bacterium *Bacillus anthracis* [1,2]. Anthrax spores are highly resistant to environmental degradation and can remain infectious for decades. In the past, human exposure to anthrax resulted primarily from cutaneous contact with infected livestock [1,2]. Recently, spores formulated to readily infect the respiratory tract were intentionally released by bioterrorists in the US [3]. This caused immediate morbidity and mortality, and raised the specter of future exposure to these long-lived spores [3].

Vaccination is the least costly and most effective method of reducing susceptibility to anthrax infection and accelerating the development of protective immunity following pathogen exposure [1]. Neutralizing Abs directed against the bacteria's "protective antigen" (PA) reduce pathogenicity by preventing anthrax toxin from binding to host cells, inhibiting the germination of spores, and improving the phagocytosis/killing of spores by macrophages [4–7]. Un-

fortunately, the currently licensed human anthrax vaccine adsorbed (AVA) requires six vaccinations over 18 months followed by yearly boosters to induce and maintain protective IgG anti-PA titers [8,9]. In some vaccinees, this immunization regimen causes undesirable side effects [9].

Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory "CpG motifs" can improve the immune response to co-administered antigens [10–12]. CpG ODN interact with Toll-like receptor 9 expressed by B cells and plasmacytoid dendritic cells [13–16], improving antigen presentation and triggering the production of chemokines and Th1 and pro-inflammatory cytokines (including IFN γ , IL-6, IL-12, IL-18 and TNF α) [13,14,17,18]. In mice, CpG ODN have been shown to boost the protective efficacy of vaccines against bacterial, viral and parasitic pathogens [19–23]. However, due to evolutionary divergence in CpG recognition between species, ODN that are highly active in rodents may be poorly immunostimulatory in primates [24–26]. Thus, pre-clinical studies to examine whether CpG ODN can accelerate and boost the immune response elicited by AVA must be conducted in a relevant primate model. This work provides evidence that co-administering GMP-grade CpG ODN with AVA to rhesus macaques increases the rapidity, titer, affinity, and protective efficacy of their resultant IgG anti-PA response.

Abbreviations: rPA, recombinant PA antigen; PA, protective antigen; AVA, anthrax vaccine adsorbed (licensed human anthrax vaccine); ODN, oligodeoxynucleotide

* Corresponding author. Tel.: +1-301-827-1707; fax: +1-301-496-1810.

E-mail address: klinman@cber.fda.gov (D.M. Klinman).

2. Materials and methods

2.1. Reagents

The phosphorothioate ODN mixture containing equimolar amounts of ATCGACTCTCGAGCGTTCTC, TCGTTCTGTTCTC and TCGAGCGTTCTC was synthesized at the Center for Biologics Core Facility. ODN 7909 was provided by Coley Pharmaceuticals. All ODN had less than <0.1 EU of endotoxin/mg of DNA as assessed by a Limulus amoebocyte lysate assay (QCL-1000, BioWhittaker). AVA was obtained from BioPort Corporation (East Lansing, MI).

The attenuated non-encapsulated Sterne strain of anthrax was obtained from the culture collection of the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD. Spores were prepared and stored as previously described [27].

2.2. Animals

All animal studies were ACUC approved and were conducted in AAALAC accredited facilities. Animals were monitored daily by veterinarians. Specific pathogen-free A/J mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed in sterile micro-isolator cages in a barrier environment.

In the first experiment, rhesus macaques (3–5 kg, $N = 6$ per group) were immunized s.c. at 0 and 6 weeks with 0.5 ml of AVA plus 250 µg of an equimolar mixture of 3 CpG ODN (described below), and then “challenged” with 10^5 Sterne strain anthrax spores when serum anti-PA titers returned to baseline at week 26. Serum Ab levels were monitored 3 weeks post-immunization and 2 weeks post-boost per challenge. In the second experiment, macaques (3–5 kg, $N = 5$ per group) were immunized s.c. with 0.5 ml of AVA plus 500 µg of ODN 7909 or the same mixture of 3 CpG ODN mentioned above. Animals were bled at least weekly for 6 weeks. All treatments were administered and peripheral blood samples obtained from ketamine anesthetized animals (10 mg/kg, Ketaject, Phoenix Pharmaceuticals, St. Joseph, MD).

2.3. Anti-PA ELISA and avidity assays

Anti-PA titers were measured by coating 96-well Immulon 2 microtiter plates (Thermo LabSystems, Franklin MA) with 1 µg/ml of recombinant PA (rPA) (produced at USAMRIID as previously described [28]). Serum samples were serially diluted in PBS plus 5% non-fat dry milk and incubated on these plates for 1 h at RT. The plates were washed, and for avidity assays overlaid for 15 min with 200 µl of 6 M urea. Bound Ab was detected using peroxidase-conjugated goat anti-human IgG (Kirkegaard & Perry, Gaithersburg, MD) followed by ABTS substrate (Kirkegaard & Perry). Absorbance values were measured at 405 nm, and Ab titers

calculated from the linear portion of the titration curve containing at least three contiguous dilutions ($R^2 > 0.9800$). Ab titers represent the reciprocal of the dilution that resulted in an absorbance value of 0.400, which was approximately twice background levels. For avidity comparisons, titers were determined by comparison to a standard curve generated using high-titered anti-PA serum. All samples were analyzed in triplicate.

2.4. Serum transfer study

Serum from all monkeys in each treatment group was pooled. One hundred microliters of pooled serum was injected i.p. into 6-week-old male A/J mice ($N = 20$ per group). The following day, mice were challenged i.p. with 500 µl of PBS containing 30 LD₅₀ Sterne strain anthrax spores. Mice were monitored daily for 2 weeks, and time to death was recorded.

2.5. Toxin neutralization assay

RAW264.7 cells were plated overnight in 96-well microtiter plates at a concentration of 30,000 cells per well. Serum from vaccinated monkeys was serially diluted in complete medium (starting at 1:20) and incubated with 100 ng/ml rPA plus 100 ng/ml lethal factor (Research Diagnostics Inc., Flanders NJ) for 1 h at 37 °C. This mixture was added to the cells for 4 h at 37 °C. Ten microliters of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 5 mg/ml in PBS, Sigma, St. Louis MO) was added for 2 h, followed by lysis buffer (25 g SDS in 50% DMF, pH 4.7) to dissolve the formazan crystals. Absorbance values were read at 570 with a reference filter of 690.

2.6. Statistical analysis

Differences in serum anti-PA Ab titers were evaluated by multiple regression ANOVA. Differences in survival were evaluated using Chi-square analysis of Kaplan–Meier curves.

3. Results

3.1. CpG ODN increase the speed and titer of the IgG anti-PA Ab response induced by AVA immunization

Initial studies examined the effect of co-administering a mixture of CpG ODN [29] with AVA on the resultant immune response. The production of serum IgG anti-PA Abs was monitored, since such Abs are necessary and sufficient to protect against anthrax infection [1]. As seen in Fig. 1, adding CpG ODN to AVA increased the primary and secondary IgG response by two- to three-fold when compared to AVA alone ($P < 0.02$). At week 26, when Ab titers had

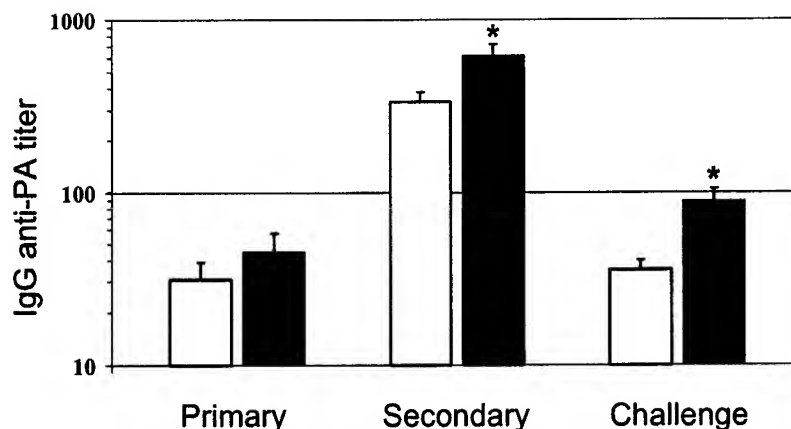


Fig. 1. Rhesus macaques (6 per group) were immunized s.c. with 0.5 ml of AVA alone (□) or combined with 250 µg of a mixture of the 3 CpG ODN described in Section 2 (■). Animals were boosted with the same material at week 6, and challenged with 10^5 Sterne strain anthrax spores at week 26. IgG anti-PA titers were measured in the serum of each animal 3 weeks post-immunization and 2 weeks post-boost per challenge. Results represent the mean \pm S.E.M. of these titers. (*) Significantly higher IgG anti-PA titer at that time point, compared to animals immunized with AVA alone, $P < 0.05$.

fallen to near baseline levels, the animals were challenged with the attenuated Sterne strain of anthrax. The resultant IgG anti-PA response was significantly higher in macaques primed and boosted with AVA plus CpG ODN ($P < 0.01$).

The quality of a vaccine-adjuvant combination is reflected by both the avidity and titer of the Abs induced. To assess the avidity of the IgG anti-PA Abs, their ability to remain bound to PA in the presence of 6 M urea was evaluated [30,31]. As expected, affinity-matured Abs induced by secondary immunization were significantly more avid than those elicited by primary immunization (Fig. 2, $P < 0.01$). The avidity of the secondary anti-PA response of macaques immunized with AVA + CpG ODN was significantly higher than that of animals immunized with AVA alone ($P < 0.01$).

Based on this evidence that CpG ODN could improve the immunogenicity of AVA, a second experiment was conducted utilizing GMP-grade CpG ODN optimized for human use. Clinical grade CpG ODN 7909 (currently un-

dergoing clinical trials for cancer therapy), the original CpG ODN mixture, or AVA alone, was administered to rhesus macaques. As seen in Fig. 3, the CpG ODN mixture again induced a stronger immune response than AVA alone. However, ODN 7909 triggered an even higher IgG anti-PA response. Starting by 11 days post-vaccination, ODN 7909 plus AVA was significantly more immunogenic than AVA alone ($P < 0.05$), generating a >3-fold higher IgG anti-PA response over the first month ($P < 0.01$).

3.2. Protective efficacy of the IgG anti-PA response

The critical measure of an antigen-adjuvant combination is its ability to induce protective immunity. Due to restrictions on the use of macaques in lethal anthrax challenge experiments, several alternative approaches were used to examine whether protective immunity was elicited by vaccination with AVA plus CpG ODN. In the first experiment,

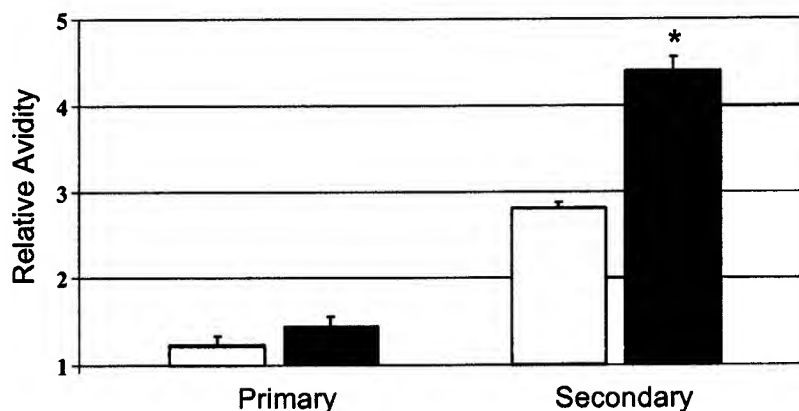


Fig. 2. The relative avidity \pm S.E.M. of the IgG anti-PA Abs in the serum of animals immunized and boosted as described in Fig. 1 was determined by elution with 6 M urea. (*) Significantly greater avidity compared to animals immunized with AVA alone, $P < 0.03$.

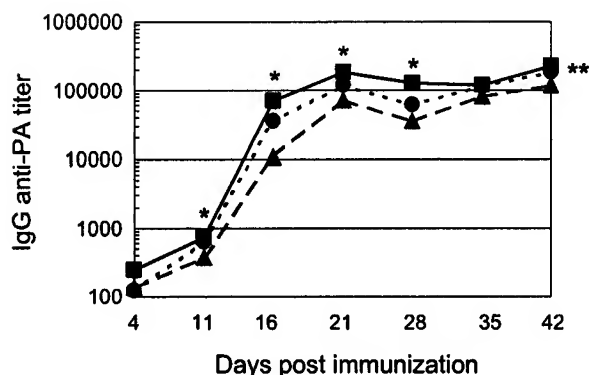


Fig. 3. Rhesus macaques (5 per group) were immunized s.c. with 0.5 ml of AVA alone (▲) or combined with 500 µg of the mixture of CpG ODN used in Fig. 1 (●) or ODN 7909 (■). IgG anti-PA titers were measured by ELISA in the serum of each animal at multiple time points post-immunization. Results represent the mean \pm S.E.M. of these titers. (*) Significantly higher IgG anti-PA titer at that time point, compared to animals immunized with AVA alone, $P < 0.05$. (**) Significantly higher cumulative IgG anti-PA titer compared to animals immunized with AVA alone, $P < 0.01$.

immunized macaques were “challenged” with the attenuated (non-lethal) Sterne strain of *B. anthracis*. Challenged animals immunized with AVA plus CpG ODN mounted a 3-fold stronger immune response than animals immunized with AVA alone (Fig. 1, $P < 0.03$).

Second, the ability of serum Abs from immunized macaques to passively protect mice from anthrax challenge was explored. A/J mice are susceptible to the attenuated Sterne strain of anthrax, but are protected by neutralizing anti-PA Abs. Serum from pre-immune or immunized macaques was passively transferred to recipient mice (20 per group). As seen in Fig. 4, animals that received pre-immune serum or serum from AVA immunized macaques rapidly succumbed to challenge by 30 LD₅₀ Sterne strain *B. anthracis* spores. In contrast, serum from macaques immunized with AVA plus CpG ODN protected nearly half of recipient mice from lethal challenge ($P < 0.03$).

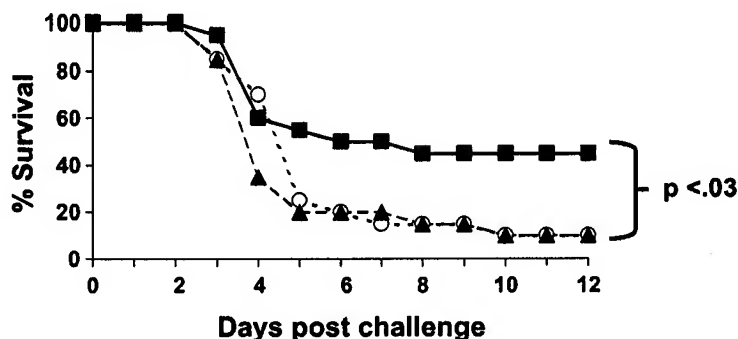


Fig. 4. Pre-immune serum (○), or serum from rhesus macaques vaccinated 11 days earlier with AVA alone (▲) or AVA + CpG ODN 7909 (■) was pooled and injected i.p. into A/J mice (0.1 ml per recipient). The following day, mice were challenged with 30 LD₅₀ of Sterne strain anthrax. Results of two independent experiments involving at combined total of 20 recipients per treatment are shown.

Table 1
Passive transfer of protection to naive recipient mice

Treatment	Donor	Recipient	Protection (%)
Serum neutralizing titer			
Pre-immunization	0 \pm 0	10 \pm 5	10
AVA alone	25 \pm 5	43 \pm 33	10
AVA + CpG ODN	434 \pm 109*	2510 \pm 1150*	45*

Rhesus macaques were immunized s.c. with 0.5 ml of AVA alone or combined with 500 µg of ODN 7909. Pre-immune serum, or serum 11 days post-vaccination, was pooled and injected i.p. into A/J mice (0.1 ml per recipient). The following day, mice were bled and then challenged with 30 LD₅₀ of Sterne strain anthrax. The serum neutralizing titer of both donors (5 per group) and recipients ($N = 20$), and percent of recipients surviving challenge, are shown. Please note that mouse serum gave a higher background than macaque serum in this neutralizing assay.

* Significantly higher than AVA group, $P < 0.05$.

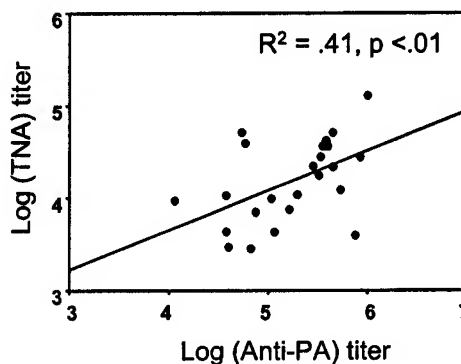


Fig. 5. IgG anti-PA titers and toxin neutralizing titers were monitored in sera from macaques immunized with AVA alone or AVA plus CpG ODN. Each data point represents the titer of a single serum measured by each assay.

These differences in susceptibility correlated with the titer of toxin neutralizing Abs present in donor serum transferred to recipient mice. Macaques immunized with AVA + ODN 7909 had on average a 17-fold higher toxin neutralizing titer than those immunized with AVA alone (Table 1, $P < 0.03$).

A similar difference in neutralizing Ab levels was detected in the serum of recipient mice immediately prior to challenge (Table 1, $P < 0.05$). As expected, serum IgG anti-PA titers correlated closely with toxin neutralizing activity (Fig. 5, $R^2 = 0.41$, $P < 0.01$).

4. Discussion

CpG motifs present in bacterial DNA and synthetic oligonucleotides directly stimulate B cells and plasmacytoid dendritic cells that express Toll-like receptor 9 [15,16]. This initiates an immunostimulatory cascade that results in the functional maturation of professional antigen presenting cells and the production of Th1 and pro-inflammatory cytokines and chemokines [13,14,32,33].

Studies in mice demonstrated that CpG ODN could serve as immune adjuvants, significantly improving the immune response elicited by protein antigens and vaccines [21,22,34,35]. CpG ODN boosted the immune response elicited by vaccines against influenza, measles, hepatitis B surface Ag and tetanus toxoid by one to three orders of magnitude, while increasing the production of Th1 cytokines and the activity of antigen-specific CTL [34–39]. Due to evolutionary divergence in TLR 9 expression between species, CpG motifs optimized for activity in humans are less effective in mice [24–26]. Thus, pre-clinical studies of CpG ODN planned for human use are best performed in non-human primates [12,24–26,28]. Studies of orangutans, aotus monkeys, and rhesus macaques showed that CpG ODN boosted the immune response elicited by the hepatitis B vaccine and heat-killed leishmania vaccine by several fold [10–12].

The current work evaluated whether clinical grade CpG ODN (developed for use in cancer immunotherapy) could improve the immune response elicited by AVA (the licensed human anthrax vaccine). Results indicate that macaques immunized with AVA plus CpG ODN mounted a stronger immune response when challenged with Sterne strain anthrax spores (Fig. 1). The combination of CpG ODN plus AVA triggered a faster, higher avidity, and higher-titered immune response than vaccine alone, resulting in a significant improvement in protective immunity against anthrax (Fig. 4).

The induction of IgG anti-PA Abs is the most relevant measure of vaccine immunogenicity, since these Abs confer protection against infection [1]. Consistent with previous studies, serum levels of IgG anti-PA Abs correlated closely with toxin neutralizing activity, a surrogate marker for protective efficacy [1]. While antigen-specific T cell and cytokine responses may also be affected by CpG ODN [14,40], reagents to monitor anthrax-specific cellular responses in macaques are not available. Yet preliminary studies in mice showed that adding CpG ODN to AVA stimulated a predominantly Th1-biased immune response characterized by increased levels of IgG2a anti-PA antibodies (data not shown).

Current results demonstrate that the avidity of the secondary IgG anti-PA immune response was significantly improved by inclusion of CpG ODN (Fig. 2). This effect is consistent with the documented ability of CpG ODN to promote the functional maturation of professional APC [41]. In this context, ongoing studies suggest that other methods of targeting AVA to professional APC also results in higher-titered, more avid immune responses.

As with all novel therapies, the possibility of adverse side effects was considered. In previous studies, CpG ODN were safely administered to rodents and primates without adverse consequences [12,42]. In the current work, no serious local or systemic adverse reactions were observed in any of the macaques treated with CpG ODN plus AVA.

Vaccines targeting biothreat pathogens are typically designed for prophylactic (pre-exposure) use. However, vaccines capable of accelerating the development of protective immunity can be of therapeutic benefit to individuals exposed to biothreat pathogens (e.g., workers in anthrax-contaminated buildings) and/or for “ring vaccination” of individuals at sites of known infection. For these latter purposes, the capacity of CpG ODN to accelerate as well as increase the titer of anti-anthrax Abs is of particular interest. Our results demonstrate that co-administering CpG ODN with AVA generates high levels of toxin neutralizing antibodies very rapidly (exceeding AVA alone by 17-fold at 11 days post-immunization). Passive transfer of these serum antibodies protected nearly half of naive mice from challenge with 30 LD₅₀ of anthrax spores (Fig. 4, $P < 0.03$ versus AVA alone). These findings support the further development of CpG ODN as an adjuvant for vaccines targeting biothreat pathogens.

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Effect of aluminum hydroxide adjuvant and formaldehyde in the formulation of rPA anthrax vaccine[☆]

S.F. Little^{a,*}, B.E. Ivins^a, W.M. Webster^a, S.L.W. Norris^b, G.P. Andrews^{a,1}

^a United States Army Medical Research Institute of Infectious Diseases, Bacteriology Division,
1425 Porter Street, Fort Detrick, Frederick, MD 21702-5033, USA

^b Goldbelt Raven, LLC/Research Support Division, 1425 Porter Street, Fort Detrick, Frederick, MD 21702-5033, USA

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Abstract

The serological response and efficacy of *Bacillus anthracis* recombinant protective antigen (rPA) vaccines formulated with aluminum hydroxide adjuvant, either with or without formaldehyde, were evaluated in rabbits. Rabbits that had been injected with a single dose of 25 µg of rPA adsorbed to 500 µg of aluminum in aluminum hydroxide gel (Alhydrogel) had a significantly higher quantitative anti-rPA IgG ELISA titers ($p < 0.0001$) and toxin neutralizing antibody (TNA) assay titers ($p < 0.0001$) than rabbits tested at the next lowest concentration of aluminum (158 µg). Rabbits injected with two doses of 50 µg of rPA formulated with 500 µg of aluminum also had significantly higher serological responses, as measured by a quantitative anti-rPA IgG ELISA ($p < 0.0001$) and TNA assay ($p < 0.0001$), than sera from rabbits injected with a rPA vaccine formulated without adjuvant. Short-term protection against an aerosol spore challenge (448 LD₅₀), however, was not significantly different between the two groups (12/12 and 11/12, respectively). Rabbits injected with a single dose of 50 µg of rPA formulated with 500 µg of aluminum and 0.2% formaldehyde had significantly higher ELISA ($p < 0.0001$) and TNA assay ($p < 0.0001$) titers than rabbits that had been injected with a rPA vaccine formulated with adjuvant but without formaldehyde. Short-term protection against a 125 LD₅₀ parenteral spore challenge, however, was not significantly different between the two groups (14/24 and 9/24, respectively; $p = 0.2476$). Under the conditions tested in the rabbit animal model, significantly higher serological responses were observed in rabbits that had been injected with rPA formulated with aluminum hydroxide gel adjuvant and formaldehyde. However, differences in short-term efficacy were not observed.

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Keywords: *Bacillus anthracis* PA vaccine formulation; Rabbit animal model; Aluminum hydroxide gel adjuvant; Formaldehyde

1. Introduction

Anthrax is an infection that may result after exposure to spores of *Bacillus anthracis* by the cutaneous, gastrointestinal, or aerosol routes and may be characterized by an extensive bacteremia and toxemia. A small number of cutaneous infections, which are usually self-limiting, as well as gastrointestinal and aerosol infections, are life threatening. The bacteremia is facilitated by the expression of a poly-D-glutamic acid capsule which interferes with phagocytosis of the vegetative bacterium. Toxemia is the result of two separate binary toxins, lethal toxin (LeTx) and edema toxin. A central component of both toxins is protective antigen (PA). After PA binds to a cellular receptor,

[☆] Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. The research described herein was sponsored by the U.S. Army Medical Research and Materiel Command, Project 02-4-CC-008.

* Corresponding author. Tel.: +1 301 619 4914.

E-mail address: stephen.little@amedd.army.mil (S.F. Little).

¹ Present address: Department of Veterinary Sciences, University of Wyoming, Wyoming State Veterinary Laboratory, 1174 Snowy Range Rd., Laramie, WY 82071, USA.

it undergoes proteolysis by the cell-surface protease resulting in a receptor-bound fragment, PA63. The PA63 molecules form a heptameric pre-pore which complexes with the enzymatic components, either lethal factor (LF), forming LeTx, or edema factor (EF), to form edema toxin. The toxin complex is then endocytosed by the cell and within the endocytic compartment the pre-pore undergoes an acidic pH-dependent conformational rearrangement that allows translocation of LF and EF into the cytosol [1].

Anthrax vaccine adsorbed, Biothrax (AVA Biothrax; BioPort Corporation, Lansing, MI) is the current vaccine licensed for human use against exposure to *B. anthracis* spores in the U.S. The vaccine is prepared by adsorbing filtered culture supernatant fluids of the V770-NPR-1 strain of *B. anthracis* to an aluminum hydroxide gel. The major protective antigen in AVA BioThrax is PA [2–5], the central component of the *B. anthracis* exotoxins. Also present in the vaccine are LF and undefined bacterial proteins, which are present in the filtered culture supernatant and which are also adsorbed onto the adjuvant [6]. In addition to the adjuvant, AVA Biothrax is formulated to contain 25 µg/ml of benzethonium chloride as a preservative and 100 µg/ml of formaldehyde as a stabilizer. Several concerns have been raised in regards to the vaccine, including the lot-to-lot variation in the amounts of PA in the vaccine [6] and occasional reactogenicity after vaccination, which may be related to the presence of uncharacterized components and possibly formaldehyde [7–9] that have served as a stimulus to develop a more fully characterized vaccine. Several studies have demonstrated the efficacy of PA in vaccines to protect against anthrax intoxication or infection [10–13]. The importance of anti-PA serum also has been shown in the identification of in vitro correlates of immunity [14–17] and in passive antibody studies [18–20]. This report evaluates the role of the aluminum hydroxide gel adjuvant and the exceptional formaldehyde in the formulation of rPA vaccines in the rabbit model using in vitro surrogate markers (the quantitative anti-rPA IgG ELISA and toxin neutralizing antibody (TNA) assay) and efficacy studies.

2. Materials and methods

2.1. Animals

An equal number of male and female New Zealand white (NZW) rabbits (3.0–3.5 kg) (Covance Research Products, Denver, Penn.) were used for each experiment. The animals received food and water *ad libitum*. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Preparation of rPA vaccine, vaccination, and challenge

Recombinant PA (rPA), expressed in a *B. anthracis* background [21,22], was manufactured as a cGMP lot by the Biopharmaceutical Production Facility at NCI-FCRC (Frederick, MD) using a modification of a reported procedure [23]. The same lot of rPA was used throughout these experiments for vaccinations and serological analysis of antibody response. LF was prepared by chromatographic separation from V770-NP1-R culture supernatants as previously described [24].

The effect of aluminum hydroxide gel adjuvant on the serological response to rPA was evaluated by adsorbing 25 µg of rPA diluted in Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS) to various concentrations of aluminum (500–15.8 µg per 0.5 ml dose in half-log dilutions) in Alhydrogel (2% Al_2O_3 ; HCl Biosector, Frederikssund, Denmark) for 20–24 h at 4 °C before use. NZW rabbits were inoculated intramuscularly (i.m.) with a single dose in 0.5 ml volumes and were bled weekly. The immunological status of the rabbits was measured by using a quantitative anti-rPA IgG ELISA and TNA assay [15].

The effect of adjuvant (Alhydrogel) on the serological response and protection was evaluated in NZW rabbits, which were inoculated i.m. at 0 and 4 weeks with 50 µg of rPA formulated with either 500 µg of aluminum per injection, final concentration, or without adjuvant in 0.5-ml volumes. Control rabbits were injected with PBS and Alhydrogel. Rabbits were bled every other week to determine serological titers to PA (quantitative anti-rPA IgG ELISA and TNA assay). At 10 weeks, rabbits were exposed to a small-particle aerosol in a modified Henderson exposure system contained within a Class III biological safety cabinet to the head only (nose and mouth) with a lethal dose of spores from the Ames isolate of *B. anthracis* [25]. Inhaled doses were calculated using the aerosol exposure concentration obtained from plate counts from the all-glass impinger which continuously sampled the test atmosphere during the 10 min exposure time and the respiratory minute volume for each animal measured by plethysmography. Spores were prepared as previously described [25]. Survival was noted for 21 days after challenge. The aerosol LD_{50} of Ames spores in NZW rabbits is 1.1×10^5 spores [25]. The average inhaled dose (average $\text{LD}_{50} \pm \text{S.D.}$) of spores was $448 \pm 214.6 \text{ LD}_{50}$.

The effect of formaldehyde on the immune response in NZW rabbits after injection of a rPA vaccine preparation was also evaluated. Vaccine preparations were formulated with 50 µg of rPA adsorbed to 500 µg of aluminum in Alhydrogel with or without 0.02% formaldehyde. Rabbits were inoculated i.m. with a single dose of vaccine. Control rabbits were injected with PBS and Alhydrogel. Rabbits were bled every other week after inoculation, and the sera were tested by a quantitative anti-rPA IgG ELISA and TNA assay. Rabbits were challenged subcutaneously (s.c.) at week 6 with 125

LD₅₀ spores from the Ames isolate of *B. anthracis* (actual count was 1.9×10^5 spores). Spores were prepared as previously described [25]. Survival was noted for 21 days after challenge. The s.c. LD₅₀ of Ames spores in NZW rabbits is 1.5×10^3 spores.

2.3. Serological analysis of antibodies

Blood was collected periodically for analysis of serum antibodies by a quantitative anti-rPA IgG ELISA and TNA assay [15]. ELISA titers were determined by interpolating the average absorbance value for triplicate wells of each sample with the absorbance values of a standard curve generated from seven dilutions of affinity purified rabbit anti-rPA IgG by linear regression analysis and reported as micrograms of anti-rPA IgG per ml (KC4 software, BioTek Instruments, Winooski, VT) [15]. Titers were presented as the geometric mean and \times/\div standard error of the geometric mean (S.E.M.). For the TNA assay, the average absorbance value of triplicate wells for each test sample dilution, less the average absorbance value of triplicate wells incubated with LeTx, was divided by the average absorbance value of control wells that contained only medium, less the average absorbance value of triplicate wells incubated with LeTx, and the ratio multiplied by 100 to obtain the percent viability of the test wells compared to the control wells:

$$\%Control = \left(\frac{\text{sample avg} - \text{LeTx avg}}{\text{medium control avg} - \text{LeTx avg}} \right) \times 100.$$

The percent control values were plotted against each respective test dilution using a four-parameter logistic equation algorithm. TNA assay titers were expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED₅₀) using XLfit software (IDBS Inc., Emeryville, CA). Titers were presented as the geometric mean and \times/\div S.E.M.

2.4. Statistical analysis

Log₁₀ transformations were applied to all ELISA and TNA assay ED₅₀ titers except for TNA assay ED₅₀ titers from results presented below in Section 3.1. After transformation, the dependent variable met assumptions of normality and homogeneity of variance. Mixed model analysis of variance (RM-ANOVA) or ANOVA were used to compare titers over time and between challenge groups. Fisher exact tests and Chi-square tests for proportions were used to compare survival rates, which are the ratio between survivors and the total number of test animals at the end of the study. Kaplan-Meier survival analysis, which is a plot of the percent survival as a function of time, was used to compare survival curves between groups. Analyses were conducted using SAS Version 8.2 (SAS Institute Inc., SAS OnlineDoc, Version 8, Cary, NC).

3. Results and discussion

3.1. Dose effect of Alhydrogel adjuvant on serological responses

During the development of an anthrax vaccine based upon rPA, numerous adjuvants were evaluated for their efficacy in guinea pigs [5,26] and non-human primates [12]. Aluminum-based adjuvants however, are the only class of adjuvants that have been approved for use in humans. AVA Biothrax is prepared from V770-NP1-R filtered culture supernatants adsorbed to 650 μg of aluminum hydroxide gel per 0.5 ml dose [6]. The British vaccine approved for human use consists of filtered culture supernatants of the Sterne strain of *B. anthracis* precipitated with aluminum phosphate gel (alum) [27]. Although the maximal amount of aluminum that is allowed for the U.S. vaccine is 850 μg per dose, the recommended maximum concentration for anthrax vaccines based upon rPA is 500 μg per dose. Our first experiment examined the serological response of NZW rabbits injected with a rPA vaccine formulated with various concentrations of aluminum present in aluminum hydroxide gel (Alhydrogel). Animals that were injected with a single dose of 25 μg of rPA adsorbed to 500 μg of aluminum per 0.5 ml dose had a geometric mean peak ELISA titer at week 2 of 31.0 μg of anti-rPA IgG per ml (Table 1). At a half-log lower dose of adjuvant (158 μg of aluminum), the geometric mean week 2 anti-rPA ELISA titer dropped significantly ($p < 0.0001$) by eight-fold to 4.0 μg of anti-rPA IgG per ml. Each half-log lower concentration of aluminum adjuvant in the rPA vaccine preparations resulted in two-fold decreases in week 2 anti-rPA ELISA titers (Table 1). The geometric mean TNA assay ED₅₀ titer at week 2 for rabbits that had been injected with a single dose of 25 μg of rPA adsorbed to 500 μg of aluminum was 360 (Table 1). The TNA assay ED₅₀ titer dropped significantly ($p < 0.0001$) by five-fold to 69.4 for rabbits injected with 158 μg of aluminum per dose (Table 1). At lower concentrations of adjuvant, a decrease in TNA assay ED₅₀ titers were also observed (Table 1). It would appear that for a maximal serological response for rabbits, the maximum recommended concentration of 500 μg of aluminum per dose was the most effective among the doses tested.

3.2. Effect of Alhydrogel on serological response and protection

The effect of the adjuvant on the serological response and protective efficacy was examined in NZW rabbits that were injected with rPA vaccines formulated either with or without Alhydrogel. Rabbits were inoculated with two doses of 50 μg of rPA at 0 and 4 weeks. For one group, rPA was adsorbed to aluminum hydroxide adjuvant at 500 μg of aluminum per dose while the second group did not receive adjuvant. Control rabbits were injected with PBS and Alhydrogel. Titers peaked 2 weeks after the second dose of vaccine on week 6 for both groups (Tables 2a and 2b). The anti-rPA IgG

Table 1

Quantitative anti-rPA IgG ELISA and TNA assay ED₅₀ titers of rabbits after a single inoculation with 25 µg of rPA adsorbed to various concentrations of aluminum

Aluminum concentration (µg)	Quantitative anti-rPA IgG ELISA titer ^a				TNA assay ED ₅₀ titer ^b
	Week 1	Week 2	Week 3	Week 4	Week 2
500	2.5 (1.409)	31.0 (1.209)	18.2 (1.158)	14.2 (1.195)	360 (1.133)
158	0.56 (1.344)	4.0 (1.372)	3.5 (1.302)	3.2 (1.294)	69.4 (1.649)
50	0.49 (1.075)	2.3 (1.251)	2.1 (1.321)	2.1 (1.217)	17.0 (1.782)
15.8	0.51 (1.272)	1.1 (1.262)	0.87 (1.233)	1.1 (1.251)	7.0 (1.641)
0	0.15 (1.213)	BLQ ^c	0.10 (1.254)	0.09 (1.085)	1.0 (1.00)

^a Titer expressed as µg of anti-rPA IgG per ml and standard error of the mean (S.E.M.) in parenthesis.

^b Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED₅₀) and S.E.M. in parenthesis. If the TNA assay ED₅₀ titer could not be extrapolated from the 4-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.

^c BLQ, below the limit of quantitation which was 0.072 µg/ml of IgG, the concentration of the lowest standard (1.44 ng/ml of IgG) multiplied by the lowest starting concentration of the sample (1:50) of the ELISA.

Table 2a

Geometric mean quantitative anti-rPA IgG ELISA titers and survival ratio of rabbits inoculated with 50 µg of rPA formulated with or without aluminum hydroxide gel adjuvant^a

Vaccination group	Anti-rPA IgG ELISA titer ^b						Survival ratio ^a
	Week 2	Week 4	Week 6	Week 8	Week 10	Post-challenge	
With adjuvant	14.1 (1.340)	8.1 (1.330)	342 (1.154)	207 (1.164)	158 (1.134)	322 (1.130)	12/12
Without adjuvant	1.5 (1.311)	1.2 (1.220)	72.0 (1.259)	50.1 (1.248)	31.8 (1.184)	511 (1.176)	11/12
Control	0.44 (1.392)	0.16 (1.616)	0.18 (1.260)	0.09 (1.138)	0.14 (1.741)	na ^c	0/4

^a NZW rabbits were inoculated i.m. at 0 and 4 weeks with rPA vaccine formulated either with or without 500 µg of aluminum adjuvant (Alhydrogel). Rabbits were challenged on week 10 by the aerosol route with spores from the Ames isolate of *B. anthracis*.

^b Titer expressed as µg of anti-rPA IgG per ml and S.E.M. in parenthesis.

^c na, sample not available.

ELISA antibody responses (Table 2a) and TNA assay ED₅₀ titers (Table 2b) were significantly higher in rabbits that were injected with rPA adsorbed to aluminum hydroxide adjuvant than in rabbits that were injected with rPA without aluminum hydroxide gel adjuvant. There was a significant difference in ELISA titers between the two groups ($F(1,108) = 161.75$, $p < 0.0001$) over time ($F(4,108) = 143.45$, $p < 0.0001$) and at each week tested (p values $p < 0.0001$ for each week). There also was a significant difference in TNA assay ED₅₀ titers between the two groups ($F(4,110) = 145.43$, $p < 0.0001$) over time ($F(4,110) = 118.60$, $p < 0.0001$) and at each week tested (p values $p < 0.004$ for each week). Protection against an aerosol exposure to *B. anthracis* spores was measured 6 weeks after the booster inoculation (week 10). Rabbits were challenged by aerosols consisting of spores of the Ames isolate of *B. anthracis*. The average inhaled dose (average

LD₅₀ ± S.D.) of spores that was measured for the rabbits was 448 ± 214.6 LD₅₀. Rabbits that were inoculated with two doses of rPA adsorbed to Alhydrogel were fully protected against the aerosol challenge (100%; 12/12), whereas 92% of rabbits (11/12) inoculated with rPA without adjuvant were protected (Table 2a). None of the control rabbits survived the challenge (0%; 0/4). The serological responses of the rabbits from each group were also compared 21 days after challenge. The post-challenge quantitative anti-rPA IgG ELISA titers and TNA assay ED₅₀ titers from the vaccination group receiving rPA adsorbed to Alhydrogel (322 µg anti-rPA IgG per ml and 6151, respectively) were similar to quantitative anti-rPA IgG ELISA titers and TNA assay ED₅₀ titers measured 2 weeks after the booster injection (342 µg anti-rPA IgG per ml and 4367, respectively). The post-challenge quantitative anti-rPA IgG ELISA titers and TNA

Table 2b

Geometric mean TNA assay ED₅₀ titers of rabbits inoculated with 50 µg of rPA formulated with or without aluminum hydroxide gel adjuvant^a

Vaccination group	TNA assay ED ₅₀ titer ^b					
	Week 2	Week 4	Week 6	Week 8	Week 10	Post-challenge
With adjuvant	203 (1.360)	74.5 (1.435)	4367 (1.125)	2029 (1.100)	1065 (1.100)	6151 (1.146)
Without adjuvant	4.5 (1.766)	2.3 (1.489)	1090 (1.218)	542 (1.226)	312 (1.235)	10422 (1.183)
Control	1.3 (1.172)	1.0 (1.00)	2.0 (1.478)	4.3 (1.626)	1.3 (1.147)	na ^c

^a NZW rabbits were inoculated i.m. at 0 and 4 weeks with rPA vaccine formulated either with or without 500 µg of aluminum adjuvant (Alhydrogel).

^b Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED₅₀) and S.E.M. in parenthesis. If the TNA assay ED₅₀ titer could not be extrapolated from the 4-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.

^c na, sample not available.

assay ED₅₀ titers however, from the rPA vaccination group formulated without Alhydrogel (511 µg anti-rPA IgG per ml and 10,422, respectively) were much higher than the quantitative anti-rPA IgG ELISA titers and TNA assay ED₅₀ titers measured 2 weeks after the booster injection (72 µg anti-rPA IgG per ml and 1090, respectively). A significant increase in ELISA or TNA assay titers suggests an absence of sterile immunity. Rabbits inoculated with rPA adsorbed to Alhydrogel had post-challenge ELISA titers and TNA assay ED₅₀ titers that were two-fold higher ($p=0.0014$) and six-fold higher ($p<0.0001$), respectively, than those measured at week 10. Significantly higher titers were measured also in rabbits that had been inoculated with rPA without Alhydrogel, which had a seven-fold increase in week 10 ELISA titers ($p<0.0001$) and a 33-fold increase in week 10 TNA assay ED₅₀ titers ($p<0.0001$) post-challenge. The difference in post-challenge ELISA titers and TNA assay ED₅₀ titers between the two groups was significantly different ($F(1,21)=5.28$, $p=0.0319$ and $F(1,21)=6.05$, $p=0.0227$, respectively). In addition to resulting in higher quantitative anti-rPA IgG ELISA titer and TNA assay ED₅₀ titers, the formulation of rPA with aluminum adjuvant in the vaccine resulted in a lower increase in the post-challenge serological responses than what was observed in rabbits that had been injected with the rPA vaccine formulated without aluminum hydroxide gel adjuvant (Tables 2a and 2b).

3.3. Effect of formaldehyde on serological response and protection

AVA Biothrax is formulated to contain 0.01% formaldehyde as a stabilizer and 0.0025% benzethonium chloride as a preservative [6]. Studies that had been conducted in evaluating rPA vaccine preparations, including serological correlates of immunity in rabbits [15], potency assay [28], and duration of immunity in rabbits [29], were not formulated with components other than aluminum hydroxide gel adjuvant. The serological response and efficacy of vaccines containing 50 µg of rPA adsorbed to Alhydrogel (500 µg of aluminum) and formulated either with 0.02% formaldehyde

or without formaldehyde were compared in rabbits inoculated i.m. with a single injection of vaccine (Table 3). Geometric mean anti-rPA IgG ELISA titers between the two groups were significantly different at week 2, ($p=0.0001$) and week 4 ($p=0.0002$) but not at week 6 ($p=0.0652$). Similarly, geometric mean TNA assay ED₅₀ titers between the two groups were significantly different at week 2 ($p<0.0001$) and week 4 ($p=0.0003$) but not at week 6 ($p=0.1119$). Rabbits were challenged s.c. at 6 weeks with spores from the Ames isolate of *B. anthracis*. The rPA vaccine formulated with 500 µg of aluminum and 0.02% formaldehyde protected 58% of rabbits (14/24) against the parenteral challenge, while 37.5% of rabbits (9/24) inoculated with rPA formulated with 500 µg of aluminum but without formaldehyde were protected. None of the control rabbits survived the challenge (0%; 0/4). The s.c. route of challenge was evaluated because it provided greater control of the number of spores that were in the challenge. Neither the difference in survival rates ($p=0.1486$), survival curves ($\chi^2(1)=1.62$, $p=0.2037$), nor mean time-to-death (4.2 days for rPA with formaldehyde and 4.5 days for rPA without formaldehyde; $p=0.7959$) between the two groups was significant. Our data do not suggest that the inclusion of formaldehyde is a necessary additive to the rPA vaccine. From these results, it appears that formaldehyde's action, in addition to acting as a stabilizer, may also be one of an adjuvant. Studies from a booster injection were not investigated.

Aluminum compounds, aluminum hydroxide (Al(OH)₃), aluminum phosphate (AlPO₄), and alum (KAl(SO₄)₂), are the only adjuvants currently approved for use in human vaccines and are used in the formulation of many veterinary vaccines. The currently licensed U.S. anthrax vaccine, AVA Biothrax, is prepared by adsorbing filtered culture supernatants of the *B. anthracis* V770-NP1-R strain to aluminum hydroxide gel [6]. The current British anthrax vaccine, AVP, is prepared by precipitating filtered culture supernatants of the *B. anthracis* Sterne strain with aluminum phosphate (alum) [27]. AVP contains more LF and EF than AVA Biothrax as measured by antibody response to these components [30]. Studies have shown that AVA Biothrax provides

Table 3

Survival ratio, quantitative anti-rPA IgG ELISA titer, and TNA assay ED₅₀ titer of rabbits injected with 50 µg of rPA formulated with or without formaldehyde^a

Vaccination Group	Survival ratio ^b	Anti-rPA IgG titer ^c			TNA assay titer ^d		
		Week 2	Week 4	Week 6	Week 2	Week 4	Week 6
With formaldehyde	14/24	53.9 (1.188)	36.3 (1.180)	12.7 (1.189)	611 (1.150)	289 (1.148)	157 (1.171)
Without formaldehyde	9/24	18.6 (1.235)	12.9 (1.217)	7.7 (1.239)	145 (1.404)	86.6 (1.367)	93.4 (1.210)
Control	0/4	BLQ ^e	BLQ	BLQ	1.3 (1.316)	2.3 (2.300)	1.0 (1.000)

^a Rabbits were inoculated with a single dose of 50 µg of rPA vaccine formulated with 500 µg aluminum adjuvant (Alhydrogel) and either with 0.02% formaldehyde or without formaldehyde.

^b Survival ratio of rabbits challenged s.c. on week 6 with spores from the Ames isolate of *B. anthracis*.

^c Titer expressed as µg of anti-rPA IgG per ml and S.E.M. in parenthesis.

^d Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED₅₀) and S.E.M. in parenthesis. If the TNA assay ED₅₀ titer could not be extrapolated from the four-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.

^e BLQ, below the limit of quantitation which was 0.072 µg/ml IgG, the concentration of the lowest standard (1.44 ng/ml IgG) multiplied by the lowest starting concentration of the sample (1:50) of the ELISA.

high-level, long-lasting protection in non-human primates [31]. Few studies have been performed on the formulation of rPA vaccines and the effect on the resulting immunological responses in animal models. Aluminum adjuvants are thought to enhance the immune response by localizing the deposition of the antigen, that desorption of antigen can occur in the interstitial fluid, and that both desorbed and adsorbed antigens are processed by antigen-presenting cells [32] and preferentially stimulate the Th2 immune (humoral) response. Anthrax vaccines formulated with either aluminum hydroxide gel or aluminum phosphate adjuvants result in comparable anti-PA titers in humans and guinea pigs [33,30]. Berthold et al. [34] reported a significant increase in ELISA titers to rPA in mice when their vaccine was formulated with either aluminum hydroxide gel or aluminum phosphate adjuvant compared to controls without adjuvant and that ELISA titers to rPA were comparable when either aluminum hydroxide gel or aluminum phosphate adjuvants were used to formulate the rPA vaccine. However, they also found that there was an optimal adjuvant concentration because at higher concentrations of aluminum hydroxide gel adjuvant, the neutralizing antibody titers decreased [34]. We did not observe a decrease in the TNA assay ED₅₀ titers in the rabbit animal model at the highest concentration of aluminum tested (500 µg). Both the anti-PA ELISA titer and toxin neutralizing antibody titers have been identified as serological correlates of immunity in rabbits and guinea pigs [13–17,25] and are thus important measurements in developing effective vaccine strategies. Various formulations have been tested in preparing anthrax vaccines based upon rPA for its ability to elicit optimal immunological responses. Recent examples include Toll-like receptor ligands CpG ODN and Resiquimod R-848 [35,36], pluronic F127, a non-ionic, hydrophilic polyoxyethylene-polyoxypropylene block copolymer [37], additional vaccine antigens such as capsule [38] or EF [39,40], DNA vaccines [41], and mucosal vaccine strategies [42,43]. That antibodies have been recognized as important in protection is demonstrated by the number of immunotherapeutic reagents that have been recently suggested [44–49]. However, protection has not always been attributed to toxin-neutralizing antibodies. Brossier et al. [50] proposed that neutralizing anti-PA antibodies may be more important in animal models that are highly susceptible to toxemia than in animal models that are more susceptible to infection. The development of a new rPA vaccine will require the identification of an acceptable aluminum compound, optimal concentration of aluminum, and approved excipients that will enhance the immunological responses necessary for protection against infection in the proper surrogate animal models.

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